Spring 5-2013

Accessing Learning in the Adult Zebrafish with a Novel Associative Learning Task

David Joseph Jouandot II
University of Southern Mississippi

The University of Southern Mississippi

ACCESSING LEARNING IN THE ADULT ZEBRAFISH WITH A NOVEL ASSOCIATIVE LEARNING TASK

by

David Joseph Jouandot II

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

May 2013

ABSTRACT

ACCESSING LEARNING IN THE ADULT ZEBRAFISH

WITH A NOVEL ASSOCIATIVE LEARNING TASK

by David Joseph Jouandot II

May 2013

The zebrafish (*Danio rerio*) is accepted in the developmental and genomic communities as a model organism. However, the capacity for the zebrafish as a behavioral model has yet to be fully acknowledged. The research presented provides evidence validating the novel task, aids in gaining a better understanding of the learning processes, and identifies individual differences. The novel associative learning task differs from any present well established behavioral model and lends itself to future development. The task provides the zebrafish community with a high output behavioral task which is readily replicated and allows one researcher to test between eight and ten fish over a period of four weeks with a total of sixteen days of actual testing. The sixteen day period consists of all three phases of testing: habitation, training, and discrimination trials. The future growth of behavioral research in zebrafish relies on the research community to develop sophisticated behavioral models for assessing the cognitive function. Behavioral models found in the rodent and avian literature can be used as a blue print to realize the full potential of the zebrafish as a behavioral model.

The University of Southern Mississippi

ACCESSING LEARNING IN THE ADULT ZEBRAFISH WITH A NOVEL ASSOCIATIVE LEARNING TASK

by

David Joseph Jouandot II

A Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Approved:

David Echevarria_____
Director

John Robinson_____

Stan Kuczaj_____

Bradley Green_____

Anthony Bell_____

Susan A. Siltanen_____
Dean of the Graduate School

May 2013

ACKNOWLEDGMENTS

The writer would like to thank the dissertation advisor, Dr. David Echevarria, and the other committee members, Dr. Stanley Kuczaj, Dr. Bradley Green, Dr. Anthony Bell, and Dr. John Robinson, for their advice and support throughout the duration of this project.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure

CHAPTER I

INTRODUCTION

The zebrafish (*Danio rerio*) is an accepted model organism in the developmental and genomic communities. However, the capacity for the zebrafish as a behavioral model has yet to be fully acknowledged. Even though there are several behavioral models currently being utilized, some models fail to apply the full complement of classical operant constructs present in the rodent and avian literature. The purpose of this reported work is to develop a behavioral task that employs classical and operant conditioning, in an effort to cultivate a more comprehensive understanding of the zebrafish's capacity for learning.

The proposed behavioral task, which is fundamentally based on Skinnerian-like operant conditioning, may aid in further progressing the zebrafish as a behavioral model. Presented here is a novel associative learning task to assess the species' capacity for learning. The study addresses three major goals: (1) to establish a simple operant task based on a three choice discrimination test previously reported in the literature, (2) to gain a better understanding of zebrafish learning, and (3) to analyze any observed differences between individuals.

The natural habitat of the zebrafish is in the flood plains, shallow ponds, or slow portions of streams in India (Spence, Gerlach, Lawrence, & Smith, 2008). They gained favor in developmental research due to a clear chorion and relatively quick development with sexual maturity being reached in approximately three months (Kimmel, Ballard, Kimmel, Wullmann, & Schilling, 1995). Once fertilized, hatching occurs between 48 and 72 hours (Kimmel et al., 1995). The relatively small size of adults (approximately 4 cm)

and their ease of housing allow a large number of them to be housed in a relatively small area (Gerlai, Fernandes, & Pereira, 2009). Because of their structural similarities and nervous system structure, zebrafish have permitted researchers to draw inferences regarding the function of the human nervous system (Carvan, Loucks, Weberb, & Williams, 2004).

CHAPTER II

REVIEW OF RELATED LITERATURE

Zebrafish Genome as a Model

One notable developmental study by Carvan et al. (2004) uses zebrafish as a model for fetal alcohol syndrome. In this study, embryos are exposed to varying concentrations of ethanol beginning four hours post fertilization and lasting 20 hours. The researchers find that embryonic exposure to ethanol results in alterations to multiple developmental systems including, but not limited to, disruption of the hypothalamus-pituitary-thyroid axis, glutamate and GABA receptor dysfunction, and suppression of growth factors (Carvan et al., 2004). The study also described the similarity of developmental deficits observed between fetal alcohol syndrome in humans and the zebrafish model (Carvan et al., 2004).

A key milestone for experimentation in zebrafish is the sequencing of the genome by the Zebrafish Model Organism Database (ZFIN), allowing researchers to readily search and explore the genome. The sequencing has also lead to a large number of mutagenic lines which are invaluable to zebrafish research. One such line is the transparent line which is used to study stem cell transplantation (White et al., 2008). There are also other lines such as the fluorescent line of zebrafish (GFP, YFP, RFP) which are designed primarily for genetic or developmental studies. The sequencing of the genome has also allowed the zebrafish to become a premiere model organism for genetic screening and as a result, models of neurological diseases have been established (Rinkwitz, Mourrain, & Becker, 2011). There are several current models of neurological disease in the zebrafish including genetic mutations and chemically induced models such

as those for both Parkinson's disease and epilepsy. One line, the *too few* mutant, has been shown to have a reduced number of 5-HT and dopamine receptors (Rink & Guo, 2004). The *too few* mutant line is created in an aim to better understand neuronal sub-groups in the zebrafish. The original study is only physiological, yet the development of the too few mutant line may have implications in behavioral research. In addition to forming behavioral profiles, researchers can use enzyme-linked immunosorbent assay (ELISA) to access neurochemical levels in the zebrafish models. Though ample research into how the aforementioned mutant line differs at the neuroanatomical level form wild type has been accomplished, the behavioral profiles of these zebrafish are relatively unknown.

The deficit in behavioral analyses of mutant zebrafish is reflective of the entire field. Along with mutants, several chemically induced models of neurological diseases have been demonstrated, but limited work has been done to characterize the associated behavioral changes of these models. For example, a model of Parkinson's disease has been established through the administration of MPTP (1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine) as described by Siegel, Agranoff, & Albers (1999) in a case study of illicit drug users who inadvertently ingested MPTP and rapidly developed pathological and clinical symptoms of Parkinson's disease. The effects of MPTP are discernible by a decrease of dopaminergic neurons (Kabashi, Brustein, Champadne, & Drapeau, 2011). In a very reductionist definition, Parkinson's disease is most often associated with impairment of voluntary movement and resting tremors. The present study could be employed to study the connection between the effects on neurotransmitter modulation with behavioral deficits. Several zebrafish models of neurological disease exist, and behavioral analyses of these paradigms would be a significant contribution to the model.

Existing studies have shown parallel effects of drugs exposure to zebrafish and humans, and there is evidence to support the use of zebrafish in developing and testing drug therapies for human diseases and disorders (Guo, 2009). This includes Alzheimer's disease, Parkinson's disease, Huntington's disease, anxiety, depression, addiction, and autism spectrum disorders. As stated by Guo (2009), "investment into this organism, to further understand its biology and disease modeling capability, and to facilitate technological build-ups, has the possibility of revolutionizing drug discovery for CNS disorders" (p. 716). Because zebrafish behavior can be studied early in development, this species can be tested for pharmacological and toxicological effects at larval and adult stages. Therefore, zebrafish are doubly useful as a tool for large scale assessment of drug effects prior to subsequent clinical trials (Rico et al., 2011). Together, these characteristics institute the zebrafish as a great adjunct to other animal models and an ideal candidate for use in high throughput screening for mutations and drug effects. Mechanisms of learning and memory are suspected to involve a large number of genes, brain regions, and neurotransmitters that would otherwise be tedious to study without a laboratory model that is amenable to high throughput techniques (Al-Imari & Gerlai, 2008).

There is an opportunity, with the zebrafish model, to not only study the behavioral effects of psychoactive chemicals on the wild type line, but also to examine differences in how disease models react to the same chemicals. Levin (2011) finds that zebrafish are "a good intermediate model between in vitro receptor and cell-based assays and classic mammalian models for drug screening" (p. 75). The future growth of behavioral research relies on the research community to create sophisticated behavioral models for assessing

cognitive function. Behavioral models found in the rodent and avian literature can be used as a blueprint to realize the full potential of the zebrafish as a behavioral model (Echevarria, Jouandot, & Toms, 2011).

Behavioral Components

The purpose of the associative learning task is to determine the ability of the zebrafish to recognize a visual stimulus and perform a goal-oriented action in response to the stimulus. The novel association task includes concepts of reinforcement, latent learning, anticipation, and locomotion. These concepts are demonstrated through a conditioned response to the stimulus. One unique component of the zebrafish model is a natural disinclination towards light. According to the literature, zebrafish display choice avoidance towards light (Lau, Mathur, Gould, & Guo, 2011). A positive association towards light is conditioned through reinforcement, which is defined as the ability of the subject to make a distinction between a particular action and a reward (Skinner, 1963; Tolman, 1948). Reinforcement is arguably the driving force behind any behavioral task in which operant conditioning as a source of behavior modification. The novel task is a relatively basic operant task which is modeled after the work of B.F. Skinner (1948) and E.C. Tolman (1948).

A large portion of Skinner's operant pigeon based studies are the first of their kind, and the insight provided by this research is instrumental in using animal models for studying behavior. In his landmark paper entitled *Operant Behavior,* Skinner (1963) states that "reinforcement in its entomological sense designates simply the strength of a response" (p. 505). The conditioning of the animal revolves around the fact that the reoccurrence of a particular behavior is directly related to the result of that behavior. A

key early example of reinforcement is applied during the experiment in which Skinner discovered shaping. The discovery of shaping took place in 1943 in an effort to demonstrate the ability of the pigeon to bowl (Peterson, 2004). Skinner further progressed shaping in a laboratory setting as described in *Superstition in the Pigeon* (Skinner, 1948), and once again in the experiment with Morse (Morse & Skinner, 1957). Shaping, as developed by Skinner, allows the experimenter to reinforce approximations towards a goal. This differs from other forms of operant conditioning in that the experimenter waits for the subject to perform a certain action then reinforces only that particular action.

Operant conditioning consists of the subject existing or operating in its environment and coming into contact with a reinforcing stimulus. The reinforcer acts to increase the instances of the operant which elicits reinforcement (Skinner, Ferster, & Ferster, 1997). As a result of a particular behavior being reinforced, the subjects increasingly continue to perform the reinforced behavior in anticipation of reinforcement. Generally, operant conditioning consists of positive reinforcement through reward. Skinner adapted rodent cages into what is commonly known as a Skinner Box, which consist of an instrument for the subject to interact with. Once the subject interacts with the apparatus the behavior is reinforced with a food reward. The behavior prior to the reinforcement becomes the operant. Operant conditioning is controlled by the actions of the subject with the desired behavior being reinforced and adverse behavior being ignored (Staddon & Cerutti, 2003).

Although there are several types of conditioning which are fundamentally similar, Gilbert Atnip’s paper on stimulus and response-reinforcer contingences provides a

simplistic description of how operant conditioning differs from other types of conditioning. Atnip (1977), in operant conditioning, on each trial the lever is inserted into the chamber and if there is no lever press within ten seconds, the lever is withdrawn and no food is delivered. A lever press is necessary for reinforcer delivery on each trial. While in Classical conditioning, in each trial the lever is present for ten seconds, followed by food delivery and lever withdrawal. Lever presses have no scheduled consequences (Atnip, 1977). Through the use of an aquatic operant apparatus, the current study acts to capture and reinforce an operant behavior in the zebrafish.

The continued use of rodents as a behavioral model for conditioning led to studies that sought to characterizes innate aspects of working with rodents. The article by Andrews, Jansen, Linders, Princen, & Broekkamp (1993), aided in clarifying strain issues in the rodent literature. The study is run using operant chambers with retractable levers and a stimulus. Although the experiment provides evidence of a difference in performance levels between the four strains, the data showed that there is relatively low variation among individual rodents within a given strain. The consistency in performance that is observed in mammalian models is not commonly observed in zebrafish behavior; therefore, the novel association task will be used evaluate the variance between subjects.

The novel association task uses operant conditioning in order to create a positive association between light and the conditioned stimulus. Though operant learning tasks have a complex multifaceted history, the task is novel in concept and design to the zebrafish model. The task is modeled from the rodent and avian literature, yet the aquatic nature of the zebrafish requires special consideration when designing the testing

apparatus. That being said, the novel task is designed to be simplistic in order to establish the ability of the zebrafish model to be reliably used in operant discrimination tasks.

Parallel to the stimulus response learning of the 1940's and 1950's, Edward Tolman demonstrated the ability to study multifaceted cognitive activity in rodents (Tolman, 1948). Tolman differed from other behaviorists of his time by taking an anti-reductionists approach to how he examined learning (Johnson & Crowe 2008). Researchers have demonstrated the effect of motivation/reward on the development of cognitive maps, as well as the ability of rodents to alter their perception of the maps in response to a change in their surroundings (Tolman, 1948). Animals cultivate expectations of their surroundings, and these anticipations affect the behavior of the animal (Johnson & Crowe, 2008). In other words, an animal that is conditioned to respond to a stimulus through reinforcement expects reinforcement after responding to the conditioned stimulus.

Current Behavioral Tasks

While behavioral research is relatively new, several tasks have been shown to assess learning in the species. The tasks described below are currently used in the behavioral analyses of zebrafish.

Predator Avoidance

It is arguable whether certain predator cues are learned or genetically ingrained (Burgess & Granato, 2007; Gerlai et al., 2009; Saverino & Gerlai, 2008). Yet, predator avoidance is considered a task in the laboratory even though it is a survival skill in nature. Gerlai, et al. (2009) demonstrates that zebrafish shy away from images of the Indian leaf fish, a natural predator. In this study, video recordings are used to measure the

following: percentage of time swimming, distance to the bottom of the tank, velocity, turn angle, and erratic movements. The zebrafish are placed in a novel tank for 20 minutes to acclimate. During that time, they are shown an image of six shoaling zebrafish. After the acclimation, the predator images are shown and the behavior is recorded (Gerlai, et al., 2009). A separate study shows that they swim towards images of conspecifics in an attempt to shoal (Saverino & Gerlai, 2008). Results of the predator task provide evidence that zebrafish can discriminate between different visual stimuli.

Conditioned Place Preference

As Lau, Bretaud, Huang, Lin, & Guo, (2006) demonstrate, conditioned place preference tasks measure the ability to orient towards a preferred location in anticipation of reward. This basic task shows the ability to make an association between location and reward through conditioning, and is essential to developing more complex tasks. In this study, zebrafish were placed in either a white chamber or spotted chamber depending on their natural preference and are exposed to either a food reward or morphine. When exposed to a reward in the form of brine shrimp or morphine, zebrafish exhibit place preference by spending more time in the chamber in which they experienced reward (Lau et al., 2006). According to Lau et al. (2006), naloxone (opioid antagonist) disrupts both morphine and food induced place preference. When naloxone is administered, along with either morphine or food, the subjects fail to establish place preference . The *too few* mutant line, which has a reduction of dopamine and serotonin neurons, does not exhibit the place preference shown in wild type (Lau et al., 2006). These findings provide evidence that genetic mutants can be a valuable tool in behavioral tasks.

Three Compartment Zebrafish Maze

Levin, Chrysanthis, Yacism, & Linney (2003) devised the three compartment zebrafish maze to condition subjects to avoid a compartment through aversive reinforcement. Aversive reinforcement, as operationally defined by Levin et al. (2003), requires that the wall of the chamber be moved inward to reduce the amount of space in the compartment and restrict movement. Zebrafish are placed in a central compartment and allowed to acclimate for 60 seconds. After the initial 60 seconds, the gates are removed and fish are allowed to swim into one of two compartments located on either side of the central compartment. If the subject swims into the left compartment, the gate is closed and zebrafish are left undisturbed for 30 seconds. However, if the subject swims into the right compartment, the gate is closed and they undergo aversive contingency for 10 seconds. After this contingency, zebrafish are allowed to return to the central compartment and repeat the trial (Levin et al., 2003). After 18 days of testing, the correct choice (the left compartment) is made approximately 64% of the time. This work provides evidence for orientation.

T-Maze

The T-maze conditions fish with a food reward to make an association between particular colors and reward (Colwill, Raymond, Ferreia, & Escudero, 2005). Fish are placed in the stem of the T-maze and are allowed to enter one of two distinct arms of the apparatus. Depending on which predetermined group the fish are in, subjects are conditioned to associate one color with food and the other without food. The work described by Colwill et al. (2005) provides evidence for the ability of zebrafish to discriminate between visual stimuli. The researchers also used vertical and horizontal

stripes as conditioned stimuli. Fish are able to differentiate between stripe patterns, showing preference to the arm paired with food reward. After training and conducting several trials, the food designated arm of the maze is chosen approximately 80% of the time (Colwill, et al., 2005). Presumably, one could argue that chance in the T-maze is 50%; so the ability to perform well above chance shows both anticipation of food reward and an orientation to find the location of the target arm of the maze. Zebrafish perform at the same level for both color stimuli and stripes; however, the learning curve for the striped compartments is longer than colored stimuli. The difference is vindicated by stating that they may have superior or preferential processing of hue (Colwill, et al., 2005).

Aquatic Plus Maze

The plus maze encompasses four identically sized square chambers fixed around a central chamber of equal size. Sison and Gerlai (2010) place zebrafish in the central compartment after an acclimation period. Then, the start box is lifted, allowing them to access all four branches of the maze (Sison & Gerlai, 2010). Zebrafish are conditioned to enter a specific branch which contains a food reward either by the stimuli of a red cue card or external visual cues. In this task, food is rewarded when the fish enter the arm of the maze containing the red cue card. During trials, the maze is rotated, forcing the subject to rely on external visual cues in the testing room, such as equipment and fish tanks, to locate the target chamber. While the zebrafish are trained with food as a reward for both the red cue card trials and the no cue trials, they showed increased performance between day one and day 20 (Sison & Gerlai, 2010). Thus, the aquatic plus maze is similar to the T-maze in regards to food motivation, orientation, and anticipation.

The tasks described above represent several behavioral paradigms which are currently used in the behavioral analysis. Predator avoidance, conditioned place preference, the three compartment zebrafish maze, plus maze, and T-maze are all valuable tasks. However, enhancement of behavioral research with the zebrafish model would benefit from a visual discrimination task similar to go/no-go tasks Figure 1.

	Acquisition	Retention	Anticipations	Correct Response	Correct Omission	Intermittent/Adjustable Stimuli	Orientation Towards Stimuli
Predator avoidance							
Conditioned place preference	X	X	X				
Three compartment zebrafish maze	X	X	X	X			
Plus maze	X	X	X	X		X	X
T-maze	X	X	X	X		X	X
Associative learning task utilizing auto shaping	X	X	X	X		X	X
Go/No-Go	X	X	X	X	X	X	X

Figure 1. Current tasks and behavioral components. Current tasks and behavioral components currently utilized in zebrafish research.

The novel task provides insight into learning capabilities of zebrafish, and is a pivotal component in developing a go/no-go task tailored for this species. A go/no-go task would demonstrate the ability of zebrafish to not only preform a conditioned response in the presence of a stimulus, but also to withhold response in the absence of the stimulus. The ability of a subject to withhold response in the absence of the stimulus in a go/no-go task is considered a correct omission (Eagle, Bari, & Robbins, 2008). Correct omission is unique to go/no-go in comparison to the current tasks described above, and could one day allow for more direct comparison between rodent and zebrafish behavioral research, as well as opening the door to more complex behavioral tasks. The ability to compare zebrafish to a well-recognized behavioral model, such as the rodent model, is a crucial step towards zebrafish emerging as a more significant behavioral model for human disease.

The Novel Task

The task's main source of inspiration came from the operant constructs by Skinner (1948) and Tolman (1948). The focus of the proposed project is identification of the acquisition process that occurs during the learning phase of the task, along with the ability to retain the conditioned response. As previously mentioned, behavioral models have thus far been limited in the zebrafish. The current task allows the researcher to assess the individual capacity for learning and retention. The experimenter has experienced that zebrafish display variable performance capabilities on operant tasks. The researcher looked at the daily performance of each subject throughout habituation, training, and trial phases of the experiment with the goal of assessing the learning capabilities of individuals. In order for the model to progress, a better understanding of the capacity for learning in individual zebrafish is required to create distinct, replicable behavioral tasks.

The novel task involves training subjects to respond to a conditioned stimulus, which requires a precise locomotor response in order to receive a reward. Response to the conditioned stimulus is achieved by reinforcing an operant behavior. The operant behavior in the novel task is the motion of swimming into the test chamber, which is reinforced through food reward. Removal of the gate separating the home area from the test chamber acts as the discriminant stimulus. The nature of using zebrafish in an operant task requires the development of an aquatic operant apparatus similar to a Skinner box. The main spatial difference between the reported task and the classic Skinner box is the presence of two distinct chambers in the current task: the home area and a test chamber. Because zebrafish lack limbs, the apparatus is designed so that

response to the stimulus is signified by movement into the test chamber opposed to a key peck or lever press. At the beginning of a discrimination trial, the fish is placed in the home area and access to the test chamber is restricted. After the onset of the trial, the test chamber is illuminated and the gate separating the chambers is lifted. The change in position of the gate is sufficient to act as the discriminant stimulus. The zebrafish is allowed to either remain in the home chamber or enter the test chamber. Upon entry into the test chamber, zebrafish is reinforced with a food reward; however, failure to respond results in a 30 second confinement to the home area before trials resume. Fish only experience one testing session per day consisting of 21 trials. Once baseline performance is established, ethanol is introduced to the task in order to demonstrate the ability of the task to be used in screens.

As mentioned previously, the novel task contains a locomotor component which is, in part, due to the design of the apparatus. This is greatly influenced by the aquatic nature of zebrafish. The subject is required to travel a distance in order to complete the task and receive a reward. In moving from the home area to the test chamber, they must pass through a circular opening in the wall dividing the home and test chambers. The opening is both circular and one inch across. The moderate size of the opening requires a specific swim pattern and the ability to navigate in a specific portion of the water column.

Throughout the literature a clear component of discrimination tasks is motivation. In similarly designed operant tasks for the zebrafish, food is used as a positive reward and motivator. The ability to restrict food intake 24 hours prior to testing and the limited quantity of reward given during trials ensures that they will be sufficiently motivated throughout the trial (Bilotta, Disner, Davis, & Haggldoom, 2005). Subjects are

conditioned to respond to a visual stimulus by moving from the home area into the test chamber. Zebrafish are conditioned to expect reward in response to the conditioned stimulus by moving through a circular opening in the partition. The novel associative learning task both evaluates and tests learning and retention capacities by measuring behavioral performance.

Previous work in the Echevarria lab has shown that acute ethanol exposure causes an increase in mobility and aggression (Echevarria, Hammack, Jouandot, & Toms, 2010). The researchers also tested for confounding behaviors such as immobility and erratic swimming. Out of all the doses screened (0.125%, 0.25%, 0.5%, and 1.0%), no dose produced erratic swimming or immobility. Moderate doses of ethanol are shown to induce aggressive displays in zebrafish but not necessarily aggression itself (Echevarria et al., 2010). These results could signify an increase in boldness which could translate into an effect on decision making in the reported task. Ethanol has also been shown to have an anxiolytic (stress reducing) effect in zebrafish, which includes increased exploration and a reduction in erratic movements (Rupert et al., 2009). These results provide evidence that the anxiolytic effects and increased boldness may increase activity and could alter performance on the novel associative learning task.

As demonstrated by Echevarria et al., 2011 in the chart below, the effects of acute and chronic ethanol exposure at several doses have been characterized using current behavioral models.

Behaviors investigated	Specific measures	Ethanol exposure concentration (acute, % volume)						Ethanol exposure concentration (chronic, % volume)					During or after exposure?	Exposure time	Authors
		0.0	0.125	0.25	0.30	0.50	1.0	0.0	0.20	0.25	0.50	1.0			
Startle reaction group Swimming/shoaling behavior	Number of squares traversed, post-startle stimulus Nearest neighbor distance, mean area occupied	✓		✓		✓	✓	✓			✓		After	Acute: 2 h Chronic: 2 weeks	Dlugos and Rabin (2003)
Aggression Immobility	Biting, chasing frequency, chasing duration, retreating frequency, retreating duration	✓	✓	✓		✓	✓						During	10 min	Echevarria et al. (2010)
Locomotor activity Aggression (inclined mirror task) Social preference Antipredator behavior Light/dark preference Pigment response	Ambulation and horizontal location Spatial distribution along incline mirror and aggressive display % Time spent near stimulus group Jumping frequency Duration spent in dark Color saturation	✓		✓		✓	✓						After	60 min	Gerlai et al. (2000)
Locomotor activity Aggression (inclined mirror task) Social preference Pigment response	Ambulation Spatial distribution along incline mirror and aggressive display % Time spent near stimulus group Color saturation	✓		✓		✓	✓						After	60 min	Gerlai (2003)
Locomotor activity Antipredator behavior	Path length Jumping; distance from predator	✓		✓		✓	✓	✓		✓			After	Acute: 60 min Chronic: 2 weeks	Gerlai et al. (2006)
Habituation to novel environment	Time spent at top of tank, frequency of transitions to top, frequency of erratic movement, frequency and duration of freezing				✓				✓				After	Acute: 5 min Chronic: 2 weeks	Wong et al. (2010)

Figure 2. Acute and chronic ethanol exposure in zebrafish.

In brief, the studies reviewed by Echevarria et al. (2011), Figure 2 provides evidence of both the anxiolytic and disinhibitory effects of ethanol on the zebrafish. However, none of the tasks in the chart evaluate a conditioned response as demonstrated in the novel associative learning task being presented. While the effects of ethanol on currently established tasks have been characterized, the behavioral differences between a conditioned task and novel task may have ramifications for the effect of ethanol on the novel associative learning task.

Despite the numerous behavioral tasks currently employed with the zebrafish model, there seems to be an absence of a parsimonious behavioral task similar to go/no-go tasks. Go/no-go tasks are characterized by the subject's ability to respond in the presence of a stimulus and withhold response in the absence of the stimulus (Eagle et al., 2008). Go/no-go tasks take a sparing approach in an effort to provide a pure measure of

performance. The novel associative learning task will provide a foundation for further development of the zebrafish model.

CHAPTER III

METHODOLOGY

Subjects and Housing

Adult zebrafish (*Danio rerio*) are individually housed in an Aquatic Habitats benchtop system. The individual housing tanks used in this study are rectangular in shape and have a volume of three liters. A five step filtration system is used, consisting of: (1) 120-micron filter pad, (2) combined moving and submerged-bed biological filtration, (3) 50-micron filter cartridge, (4) activated carbon adsorbs volatile organics and other contaminants, (5) UV disinfection dose of 110 mJ/cm^2 at the beginning of the lamp. Water used in the study is tap water which runs through a 25 micron matrix filter before it is filtered through an AquaFX Barracuda four-Stage R/O DI Systems with Chloramine Blasters from Aquatic Habitats. The initial water purification and the filtration system integrated into the housing system allow for an optimal living environment which eliminates environmental stress. Unfortunately, the reverse osmosis water purification process removes the essential minerals along with contaminants. In order to provide zebrafish with an optimal environment, aquarium salts are added to the water at a rate of one tablespoon per every five gallons of water to replace essential minerals and electrolytes. Some key ions such as magnesium, potassium, sodium, and chloride are absorbed through the gill and are essential for optimal fish health. Water temperature is kept between 28 and 30 degrees Celsius. Both male and female subjects will be used in the experiments. Subjects will be kept on a 14 h on and 10 h off light cycle and food will be restricted 24 hours prior to testing. Food restriction consists of the fish being fed only a minimal amount of flake food immediately after they are run. Fish are not fed between

experiments. The task is appetite based, so it is important that the fish are hungry at the start of every trial. Female zebrafish release their eggs daily, approximately 30 minutes after sunrise, which in the laboratory is 8:00 am. The experiments did not begin before 9:00 am at which point the female fish should be finished with their daily gestational cycle. All testing is completed by approximately 2:00 pm so that testing takes place before the female fish start the buildup of eggs once again. Testing in this window should minimalize any behavioral differentiation between male and female zebrafish.

Testing Apparatus

The apparatus consists of a five gallon glass aquarium which is compartmentalized into a home area and test chamber which can be illuminated. Both chambers measure 12.1 cm long by 6.7 cm wide and 7.9 cm tall. There is also a rear compartment which contains a heater and a water circulator. The heater keeps the temperature constant in the testing apparatus, and the circulator mimics the flow of water that the fish experience in the aquarium housing. The testing apparatus consists of a home area and a test chamber separated by a divider with a circular opening that is one inch across. This opening allows the fish to gain access in and out of the test chamber from the home area. Access to the test chamber is regulated by a transparent gate which is raised and lowered by a hand operated pulley mechanism operated by the researcher at the back of the testing apparatus. The testing apparatus is designed so that the gate fits inside of the divider. The rationale behind this design is to limit the disturbance of the water in the testing apparatus. If the gate is raised or lowered on the outside of the partitioning apparatus, the movement can cause ripples in the water alarming the zebrafish. The hollow center design minimizes water disturbance inside the apparatus and reduces any

affect that the researcher's operation of the apparatus may have. The test chamber will be illuminated by a light positioned at the back of the chamber and controlled with a switch in the control panel. The control panel also contains the lever which is attached to the pulley system that operates the gate. The lever assures that the gate opens and closes properly and prevents experimenter error such as opening the gate too far or slamming the gate closed. When the lever is in the up position, the gate is closed; and when the lever is in the down position, the gate is open.

Habituation

Habituation consists of a subject being placed into the home area of the testing apparatus after 24 hours of food restriction. The subjects are food restricted during habituation in order to match the physiological conditions experienced during the training and trail phases. During the habituation period, the zebrafish are allowed to move freely through the testing apparatus with the gate opened allowing access to the test chamber. The habituation session lasts for 20 minutes. After the habituation session, they are returned to their housing aquarium and fed. After the initial five minutes in the testing apparatus, the researcher will record the number of times the zebrafish enters the test chamber. Once the final 15 minutes of the habituation session have elapsed, the total number of entries is recorded. Habituation will continue for a second day before the training process begins. The purpose of the habituation process is to both introduce the zebrafish to the test apparatus and to provide insight into the initial reaction to the novel environment.

Training

A training session consists of a five minute habituation period, after which the researcher waits for the subject to return to the home area and closes the gate. Following a ten second delay, the gate is opened and they are allowed to enter the test chamber. Once the subject enters the test chamber, the gate is closed and the light is turned on. At this point, the zebrafish receives a food reward (five-ten brine shrimp) and is given 30 seconds to consume the reward. After 30 seconds elapses, the light is turned off and the gate is opened, and the fish returns to the home area. This process is repeated until a given zebrafish makes a total of 20 entries, or the time in the testing apparatus exceeds 45 minutes. In either case, the session is terminated. Training takes place over a period of three days.

Discrimination Trials

Once the training phase is complete, the discrimination trials begin. A discrimination trial begins in the same manner as the habituation or training sessions with the subject being placed in the home area with the gates opened for five minutes. After five minutes of moving freely through the testing apparatus, they are allowed to return to the home area and the gate is closed. After a ten second delay, the test chamber is illuminated and the gate is opened. At this point, the zebrafish has the ability to either enter the test chamber (completed trial) or remain in the home area (failure to respond). The failure of a subject to respond to the conditioned stimulus may occur for several reasons. The most common could be the lack association between the conditioned stimulus and reward, yet others include: lack of motivation, appetite, sensory processing, mobility, or disinclination to light. Sensory processing, mobility, and disinclination to

light are all addressed with a fish completing a discrimination trail; the act of moving towards an illuminated chamber and consuming a live food reward. Though the protocol aims to control for some of these variables, experimental error as well as physiological anomalies in zebrafish could cause a failure to respond to the conditioned stimulus. A correct response denotes a completed trial in which the subject enters the illuminated test chamber. Upon entering the test chamber, the gate is closed and the light remains on while the subject is given the food reward. After 30 seconds has elapsed, the gate is opened and the fish returns to the home area. If the zebrafish fails to enter the illuminated test chamber after 60 seconds a failure to respond has occurred, the researcher closes the gate and the light is turned off in the test chamber while experiencing a 30 second delay before trials resume. The trials continue until the subject completes a total of 21 consecutive trials. The experiment will run four days a week for four weeks for a total of sixteen days of testing.

Drug Administration

The primary purpose of the reported research is the development of a replicable and manageable visual discrimination task; yet, the researcher is aware that corroboration of the task will open the door for the introduction of drug screenings to the task. Therefore, a basic drug treatment has been included in order to demonstrate the utility of drug treatment in the proposed task. In an effort to simply demonstrate the ability to incorporate drug screenings into the task, the researcher administered several doses of ethanol. The Echevarria lab has done a significant amount of work with ethanol in regards to stress and boldness. The current research adds to the next chapter of what is known about how ethanol affects behavior.

During acute ethanol exposure, subjects are exposed to 0.0625%, 0.25%, 0.50%, and 0.75 of ethanol per volume during testing (Echevarria et al., 2010). The ethanol doses are representative of the concentration of ethanol in the water used during testing Figure 3.

Week 1			
Day 1	Day 2	Day 3	Day 4
Habituation Day 1	Habituation Day 2	Training Day 1	Training Day 2
Week 2			
Day 5	Day 6	Day 7	Day 8
Training Day 3	Discrimination Trials 1	Discrimination Trials 2	Discrimination Trials 3
Week 3			
Day 9	Day 10	Day 11	Day 12
Discrimination Trials 4	Discrimination Trials 5	Discrimination Trials 6	Discrimination Trials 7
Week 4			
Day 13	Day 14	Day 15	Day 16
Discrimination Trials 8	Discrimination Trials 9 & Drug	Discrimination Trials 10	Discrimination Trials 11 & Drug

Figure 3. The testing schedule and drug administration for a given zebrafish.

Determination of Blood Alcohol Concentration

Blood alcohol concentrations (BAC) are measured using a spectrometer, after acute exposure to the following doses: 0.0%, 0.0625%, 0.125%, 0.25%, 0.5%, 0.75%, and 1.0%. We chose these doses in an attempt to define a range that encompasses what is generally reported in the literature. In order to quantify how much ethanol is absorbed, the project used the EnzyChrom Ethanol Assay Kit (ECET-100) from BioAssay Systems. Twelve fish per treatment group (dose) are exposed to a condition for 5, 15, or 30 minutes. Immediately afterwards fish are anesthetized with MS-222 (Tricane) and blood is extracted for processing. It is important to note that twelve fish yielded one sample with approximately 50-70 microliters of blood, or one data point.

Performance Measures

Performance measures are twofold. First, the ability to correctly respond to the stimulus before and after drug treatment is compared. Second, the researcher looked at the performance of each fish on the trial phase of the task compared to the habituation and training data in an aim to explain any difference in performance abilities for that individual fish.

Habituation is defined as the number of times in which the zebrafish enters the test chamber during the habituation phase of the task.

Training activity is measured by the number of times the zebrafish enters the test chamber and receives a food reward in the presence of the stimulus.

Statistical analyses are performed using repeated measures ANOVA and ANOVA.

CHAPTER IV

ANALYSIS OF DATA

Results

These results provide evidence for the strength of the association between stimulus and response which is developed as a result of the novel association task. Similar tasks in the rodent literature show that between subject performances has low variability; however, variable performance between individuals is becoming increasingly more evident in the zebrafish (Andrews et al., 1995). During the preliminary data exploration of 32 zebrafish tested on the novel association task, the researcher finds that subject performance can be readily grouped into three categories: poor, moderate, and high performance (Figure 1).

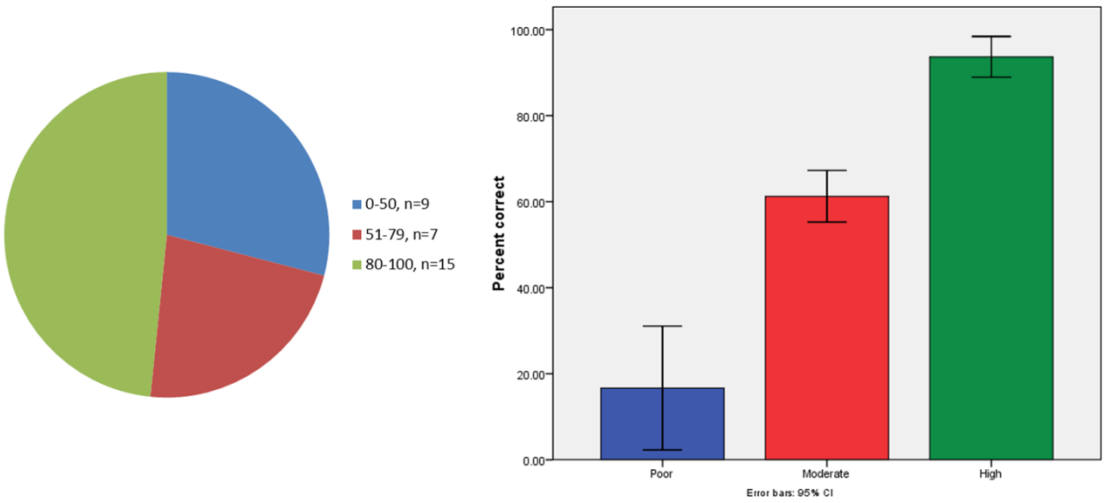

Figure 4. Group breakdown by performance. The pie chart depicts the number of individuals which had a final task performance in one of three ranges. Zebrafish are categorized into one of three groups based on their performance mode over the 8 days of the trials phase, which is measured in percent correct: (1) 0-50 n=9, (2) 51-79 n=8, or (3) 80-100 n=15.

The high performers consist of the 15 subjects that correctly respond to the stimulus more than 80% of the time. The moderate performers, the eight subjects

correctly responded 51% to 79% of the time, and the poor performers, the nine subjects that responded correctly less than 30% of the time. This provides evidence of two key components of behavioral research using zebrafish. First, there are individual performance differences between subjects. Second, the variation in performance provides evidence that some type of learning is required for the task to be successfully completed. If the task could be completed without an association being made all subjects would have equal performance. The novel association task differs from other tasks which require no training or learned behavior. In these types of tasks, the capacity for learning of individuals is not assessed. This key difference requires the present research to be analyzed in a different light than other tasks.

The performance cutoffs for the three groups are an arbitrary measure which was chosen by the researcher. The cutoff for high performance is based on operant studies in both rodents and zebrafish which report acquisition as 80%. Moderate performance is anything above chance, which given the nature of the apparatus is 50%, and poor performance is below chance. Mode was chosen as the measure which is used to make the categorization in an effort to find a measure which is minimally influence by the variability in zebrafish performance. The researcher recognizes that these performance cutoffs and measures are not without flaws. However, the measures described above provide ample means for categorizing individual task performance.

A hallmark of zebrafish being a relatively new behavioral model is the lack of replication. The development of the current task derives from the inability to replicate previously reported behavioral paradigms in the model. Therefore, during development of the current task the ability to be replicated is seen as a necessary aspect of the task. The

ability of the task to be replicated is tested by dividing the subjects into four groups (two researchers) and comparing the task performance on the last day of trials between the four groups.

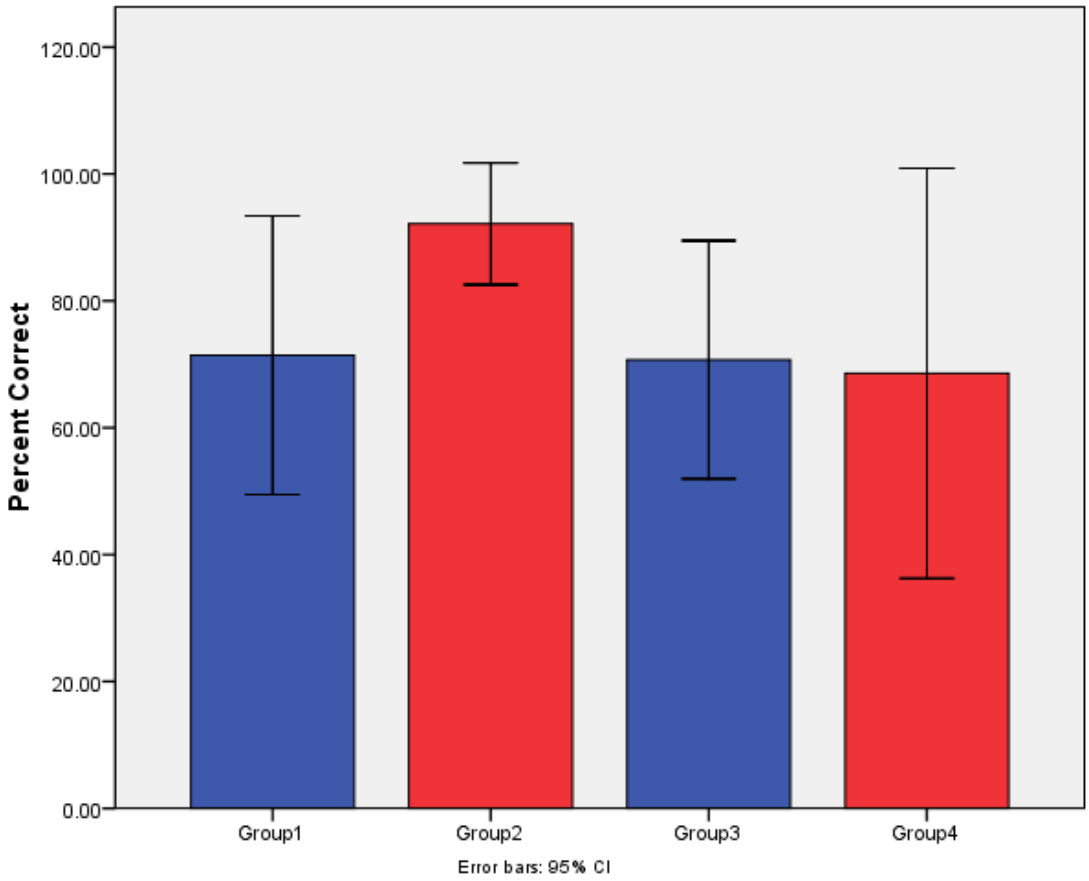

Figure 5. Comparison of the four separate running groups. Groups one and three are run by the primary researcher while groups two and four are run by a research assistant. The values are an average of fish mode over the 8 days of discrimination trials for each group.

The experiment consists of four separate groups chosen at random and tested on two apparatuses which are identical. In an effort to determine any difference between experimenter effects, a correlation is run using Pearson's R between all four groups. There is no significant difference between the groups, Figure 2. This data provides evidence that the present task can be readily replicated.

Within the evaluation of overall task performance the researcher finds it paramount to explore individual components of the conditioning process. Behavioral conditioning in the novel associative learning task consists of three phases: habituation, training, and discrimination trial. Graphical and statistical analysis is conducted on each component of the behavioral conditioning in an effort to shed light on the learning/acquisition process. The researcher also looked for a connection between the habituation and training phases and the trial phase. The habituation phase consisted of exposure to the testing apparatus for the first time. The subject is placed into the testing apparatus and given unrestricted access. Activity during the habituation phase is measured by the number of times the zebrafish moved from the home area to the testing chamber. As seen in Figure 3, the researcher finds that the activity level of individuals varied with an average activity of 11.4 over the two days of habituation.

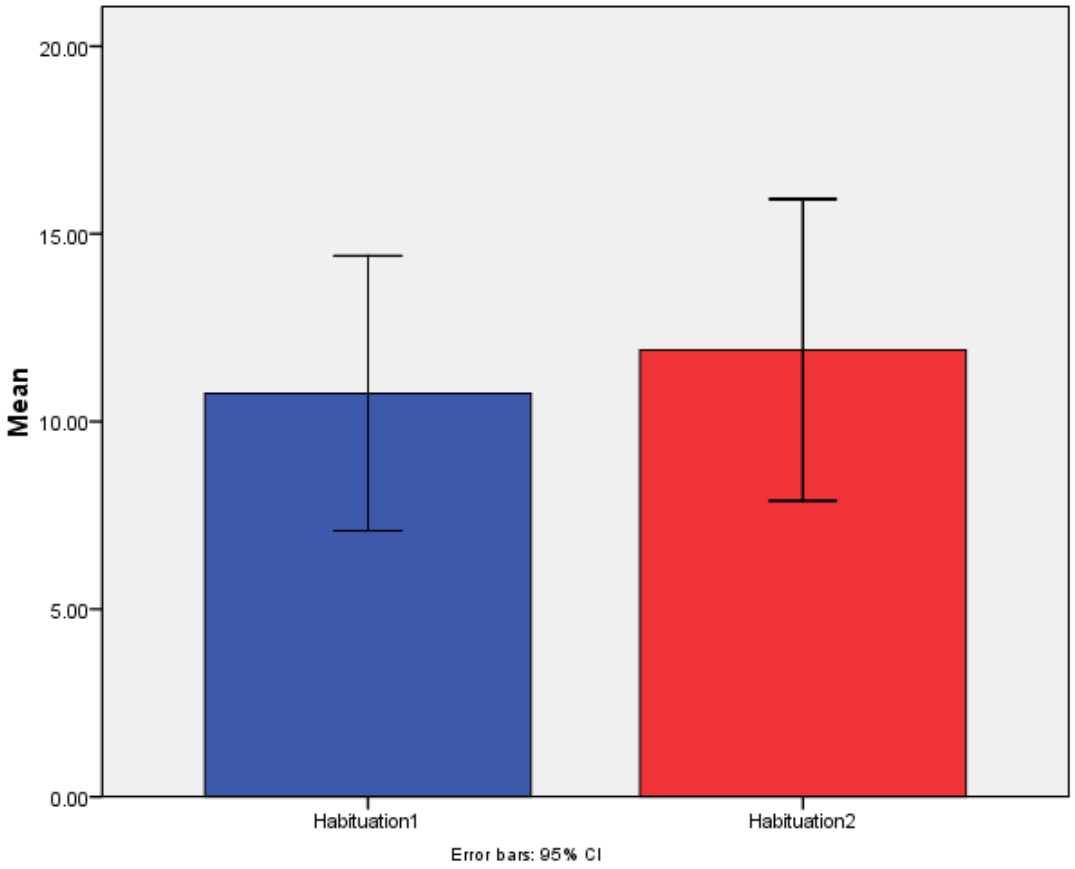

Figure 6. Habituation day one compared to day two for all subjects n=32. The average activity for habituation on day one is 10.75 with the average for day two is 12. Statistical analysis showed no significant difference between days one and two.

The lack of significance and variation suggests that activity levels are constant and distinct for individuals. Though it is important to understand the habituation process, the purpose of habituation is to familiarize the animal with the novel environment in an effort to minimize stress during the training phase. Due to the lack of conditioning in the habituation phase, no significant difference in activity is expected between days one and two.

The training phase takes place over a three day period and immediately follows the habituation phase. The training phase captures operant behavior to condition a response linking the stimulus to the reward. Briefly, the zebrafish is given a food reward

in the presence of an illuminated test chamber (conditioned stimulus). The researcher uses food reward to create a positive association towards the visual stimulus. Performance for the training phase is measured by how many times the zebrafish enters into the test chamber and receives the food reward. Though this measure is similar to the activity measure of the habituation phase, in the training phase activity is reinforced with the food reward. As depicted in Figure 4, days one and two of the training phase have an average activity of 11.1 with day three having an average performance of 11.4. Statistical analysis shows no significant difference between activity levels for any day of training. Though conventional thinking may assume that the zebrafish would become more active over the duration of the training phase, the absence of an increase in activity levels over the three day training period suggests a type of consistency in behavior. Because the habituation and training phase provides insight on how zebrafish behaviorally adjust to a novel environment, the next step is to evaluate the trial phase of the experiment.

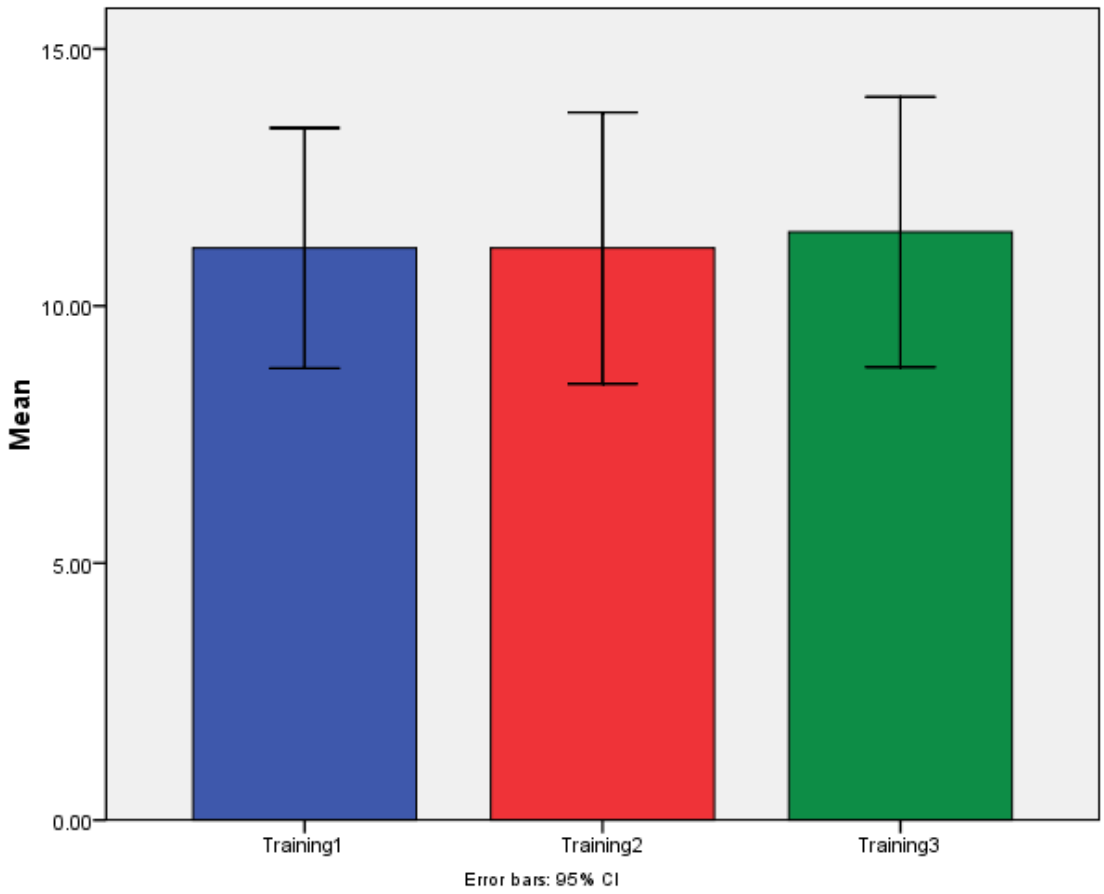

Figure 7. Comparison for training days one, two, and three for all subjects n=32. Days one and two of the training phase had an average activity of 11.1 with day three having an average performance of 11.4. There is no statistical difference between activity levels for any day of training.

The experiment consists of a total of sixteen days with days six through sixteen being the trial phase, with drug administration beginning on day fourteen. The current data are the experimental results for experimental days six through thirteen, which are the first eight days of the trial phase. The trial phase consists of the subject receiving a food reward as a result of a directed locomotor response to the visual stimulus. Performance is recorded in percent correct with the failure to respond being signified by the lack of response towards the conditioned stimulus after a period of 60 seconds. Of the 32

zebrafish tested, 23 are responding with a mode over the eight days of trials over 50%. Fifteen of the subjects reached a performance level of more than 80% Figure 5.

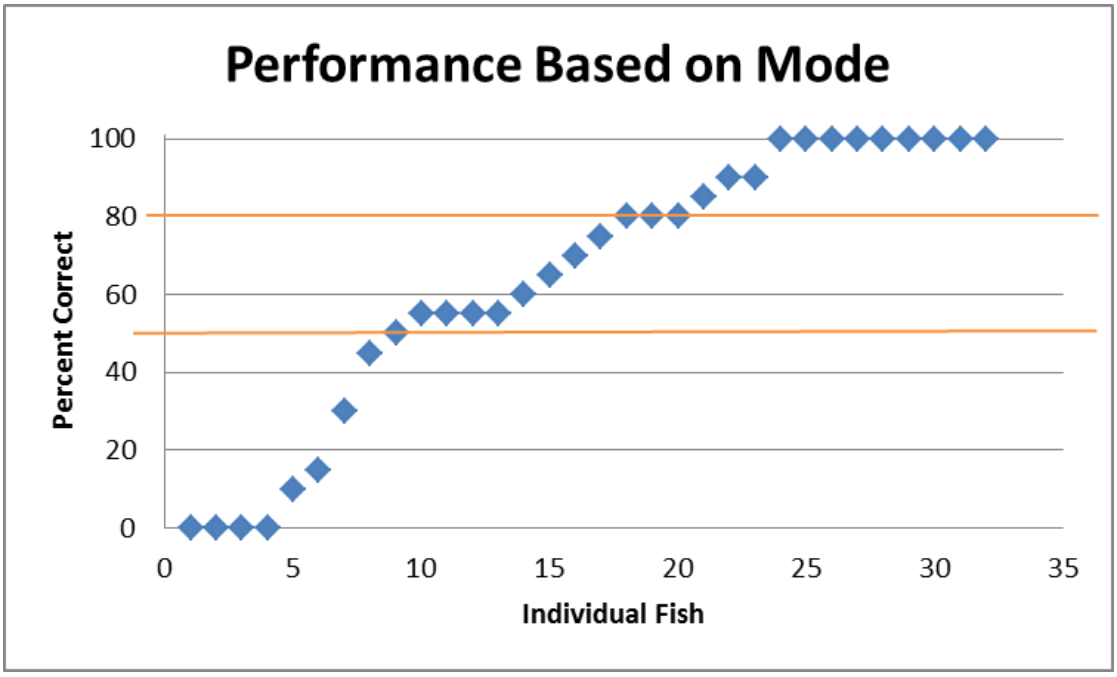

Figure 8. Performance based on mode over the eight days of trials. The graph depicts a scatter plot of all 32 subjects' performance mode over 8 days of the trial phase.

As seen above, the performance of individuals has variability uncommon to other species on similar tasks (Andrews et al., 1995). The use of the most common response aids in controlling for variation in performance, while providing a representative measure of task performance. In an effort to better understand the acquisition process and variability, the researcher looked at the trial performance for individual zebrafish figure (9, 10, and 11).

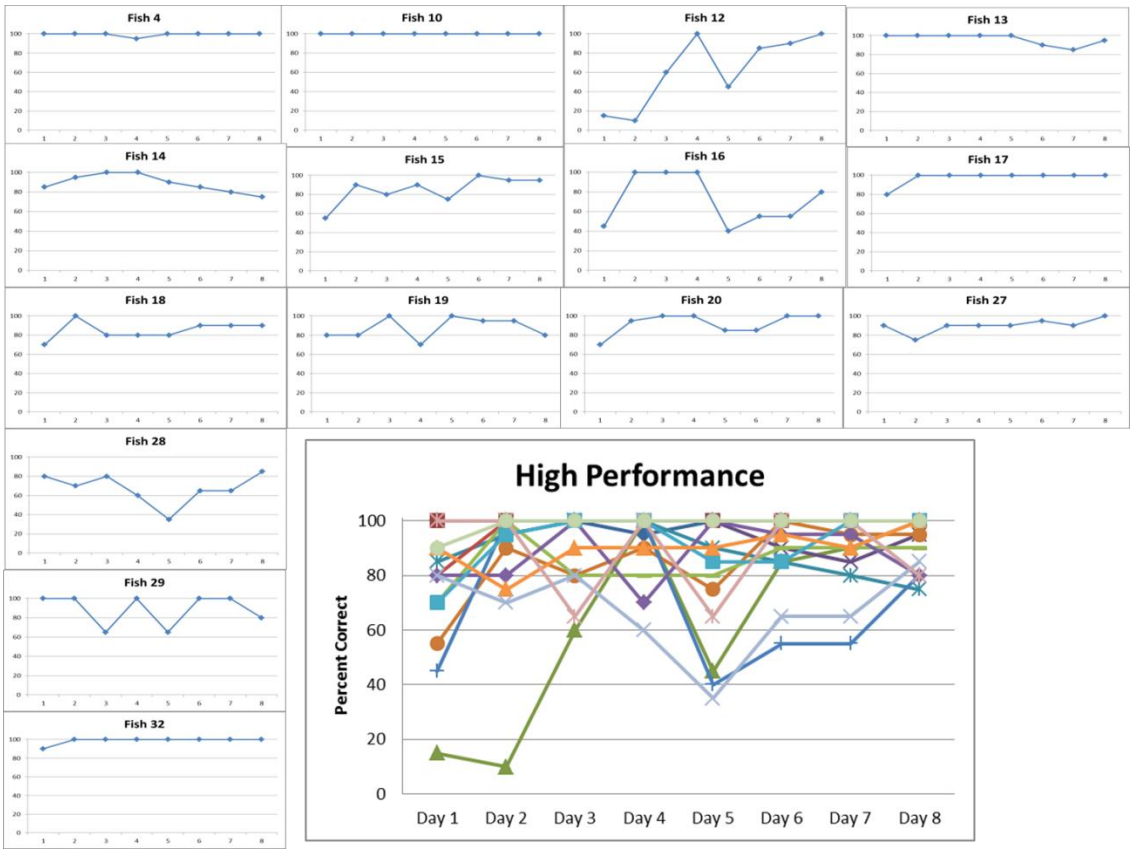

Figure 9. Individual performance over eight days of trials for high performers. The graphs above demonstrate individual performance data for the 15 subjects performing with a mode at or above 80% correct. The data represents all eight days of discrimination trials.

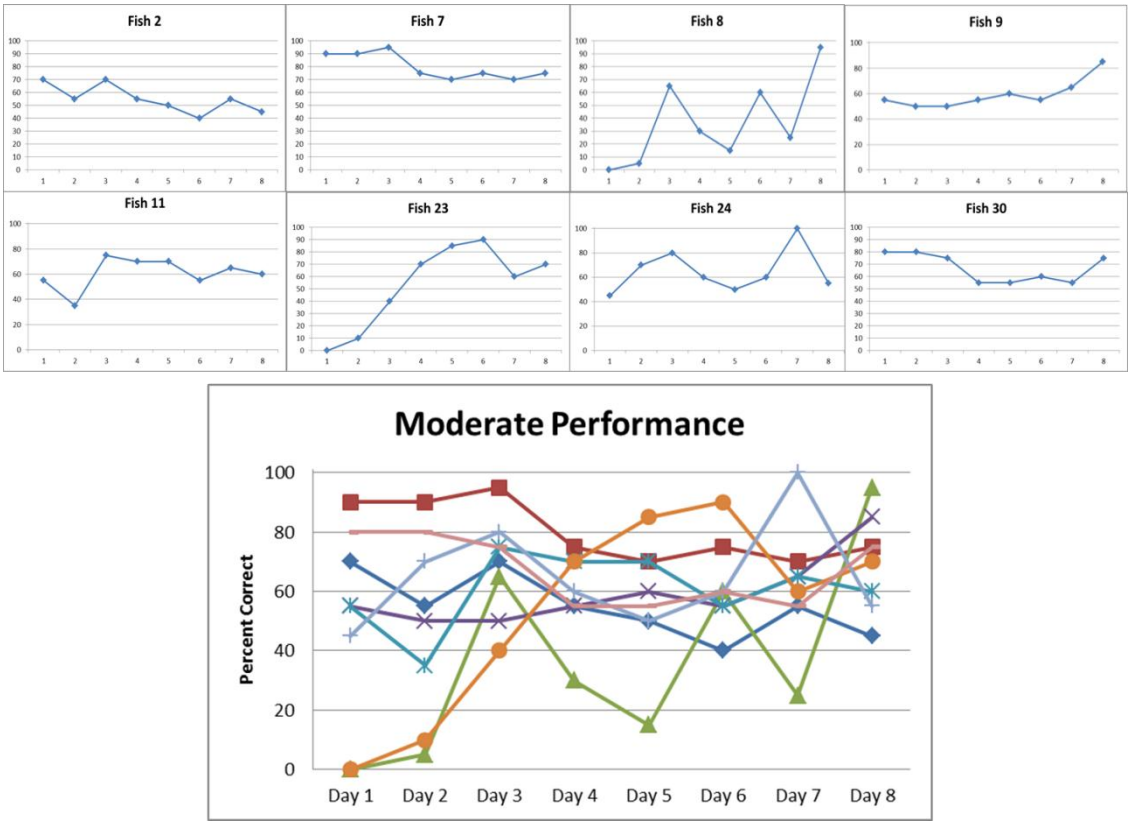

Figure 10. Individual performance over eight days of trials for moderate performers. The graphs above demonstrate the individual performance data for the eight subjects performing with a mode between 51% and 79% correct. The data represents all eight days of discrimination trials.

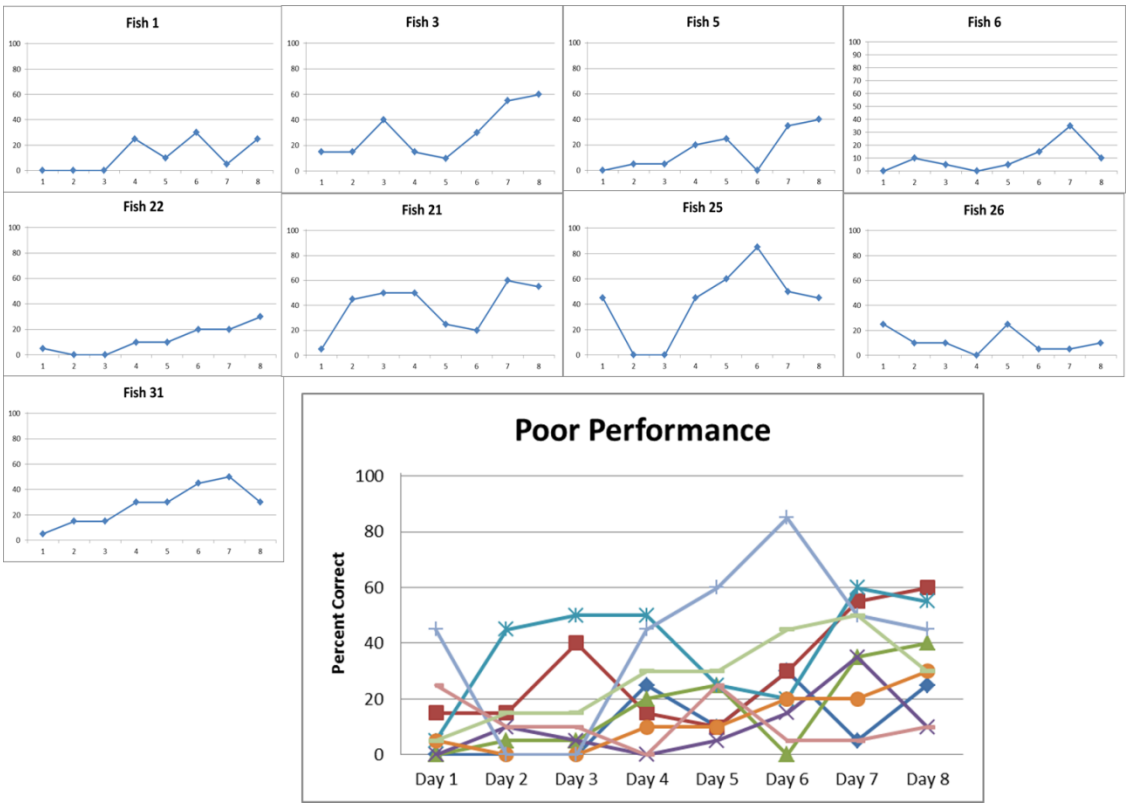

Figure 11. Individual performance over eight days of trials for poor performers. The graphs above demonstrate the individual performance data for the nine subjects performing with a mode below 50% correct. The data represents all eight days of discrimination trials.

The researcher finds that during the trial phase any individual falls into one of three general categories. First, the data for the majority of the subjects suggests that the association between the stimulus and food reward is made during the training phase. For those fish, the trial phase acts merely to reinforce or strengthen the conditioned association. Zebrafish in this group at the onset of the trial phase present with a performance above 60%, with a large number of individuals in the group responding to the stimulus more than 80% of the time. The second, smaller group presents at a relatively lower performance at the onset of the trial phase, but gradually improves performance over the eight days of trials. The data for these subjects presents in a manner that is typically considered a learning curve. During the trial phase, the association

between stimulus and reward is not only strengthened, but also, in some cases, developed. The third and smallest group of the three is signified by subjects that present with low performance when compared to group one and fail to either strengthen or create the association between stimulus and reward. The variation in the ability of an individual to make the association between stimulus and reward brings to light an aspect of using zebrafish in behavioral tasks, which had previously not been characterized. Now that variation has been identified as a characteristic of the model, the researcher continues to explore the performance of all thirty two subjects.

When analyzing the data points of individual subjects, the results from the current study continuously differ from what in observed in traditional learning. Conventionally when learning a new task animal species normally present with poor performance at the onset of trials with increasing performance during the learning phase. Conversely, once the task has been acquired performance becomes consistent. With the exception of a few subjects, zebrafish do not follow a typical learning curve on the current task.

Though data on individual performance aids in understanding the acquisition process of individual zebrafish, the second part is to determine how individual performance reflects the performance of the subjects as a group. A repeated measure ANOVA is run comparing day one of trials to all subsequent days. The analysis shows a significant difference between day one of trials compared to days three, four, six, seven, and eight Figure 9. Day one to three $p = 0.008$, one to four $p = 0.011$, day one to six $p = 0.007$, day one to seven $p = 0.002$, and day one to eight $p = 0.001$. This data provides evidence that by day three of the trial phase, the subjects have made a correlation between stimulus and response, and with the exception of the performance lag seen on

day five, the conditioned response is stronger than the onset of the trial phase. The data suggests that by day three of the trial phase, an association between stimulus and reward, which is significantly greater than the association at days one and two, has been established.

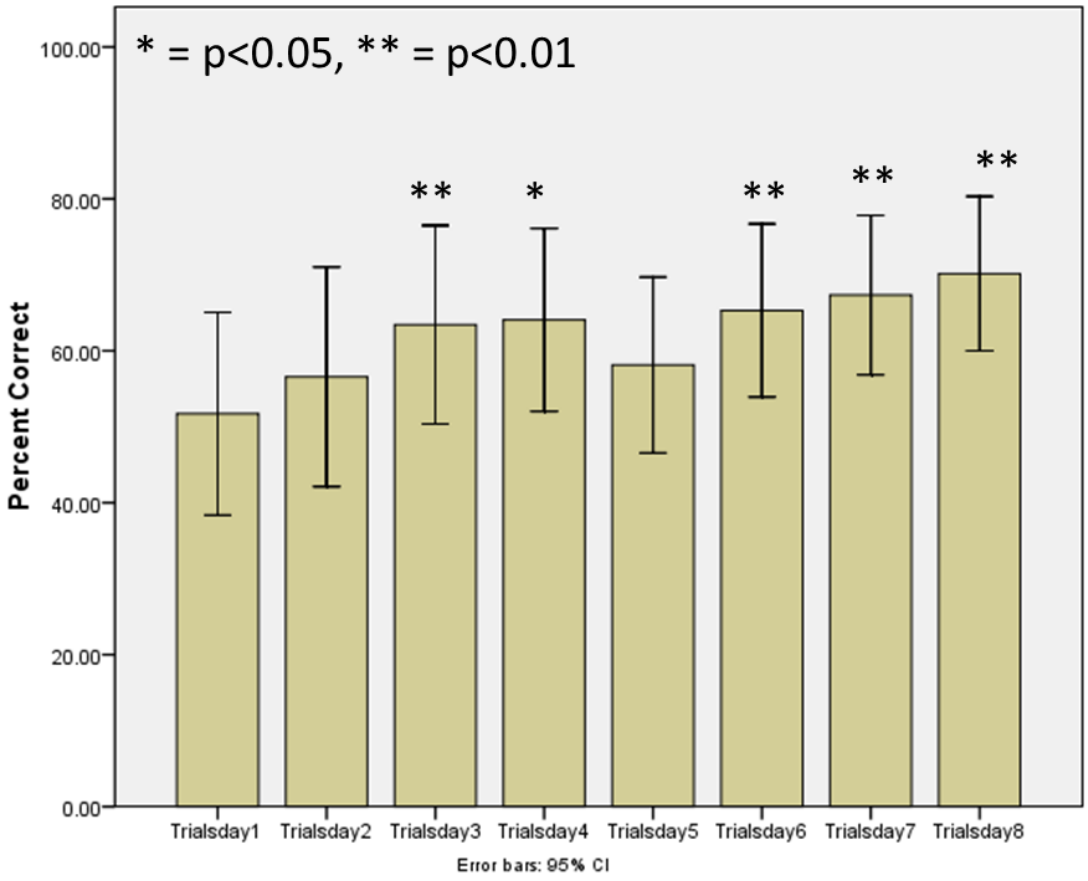

Figure 12. Trial performance for all n=32 subjects comparing each trial day to day one. A repeated measures ANOVA showed a significant performance increase between day one of trials compared to days 3, 4, 6, 7, and 8.

In an effort to characterize task acquisition based on trial performance, the research categorized the zebrafish into one of two groups. Performance on the final days of trials is used as the cutoff for group determination, ether above or below 80% correct. Due to sample distribution, the 80% cutoff is established in order to maintain statistical

power and provide equal sample size. Figure 10 demonstrates that separating zebrafish by final trial performance, subjects in the above 80% group show a significant increase in performance by day three compared to day one. However, individuals in the below 80% group do not show an increase in performance until day seven compared to day one.

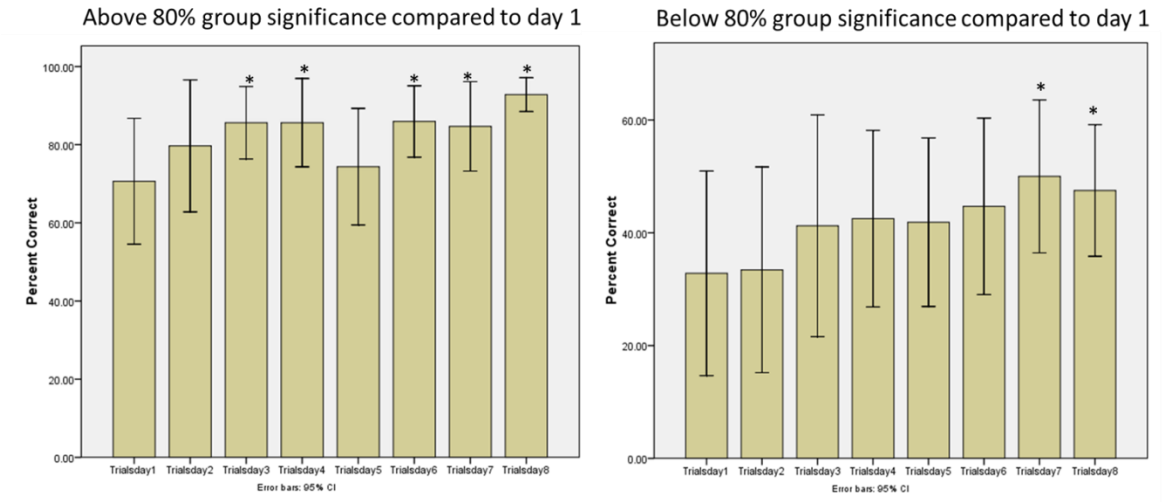

Figure 13. Trial performance comparing each trial day to day one with subjects being grouped by performance. A repeated measure ANOVA showed a significant performance increase between day one of trials compared to days three, four, six, seven, and eight for the above 80% group. Yet, the below 80% group only display a significant performance increase on days seven and eight when compared to day one.

While analyzing the data, the question arose whether or not it would be possible to utilize the habituation or training data as a means to predict performance. The ability to predict performance early in the experimental procedure would allow researchers to select a given performance range based off habituation and training data. This would allow the elimination of subjects that are not expected to perform in that range without having to complete the eight day long trial phase prior to testing a psychoactive compound. For example, when testing a compound that is thought to have cognitive enhancing properties, it would suite the researcher to test subjects that are displaying a low level of performance on the task. Though one may presume that a zebrafish with low

activity levels in the habituation and training phase would have a low level of performance on the task, statistical analysis fails to provide evidence of a reliable predictor of performance (data not shown). Developing screens based on habituation and training data may aid in predicting performance; however, the variability observed in the species prevents this researcher from proposing a screening method at this time.

In the development of this novel association task, the primary focus is on the reliability of the task as well as establishing an understanding of the acquisition process of the subjects. Nevertheless, the researcher also acknowledged the potential for the task to be used as a means of testing the effects of psychoactive compounds. In the current experiment testing of psychoactive drugs is designed to provide an additional analysis to demonstrate the potential of the task for drug testing. The researcher chose to test the effects of acute ethanol exposure on task performance.

Acute Ethanol Exposure

The first set of experiments explored the amount of ethanol which is actually absorbed into the zebrafish. To achieve this, the researcher measured the blood alcohol concentration at several doses and time points. Subjects are exposed to all six doses (0.0%, 0.0625%, 0.125%, 0.25%, 0.5%, 0.75%, and 1.0%) over three time points: 5, 15, and 30 minutes.

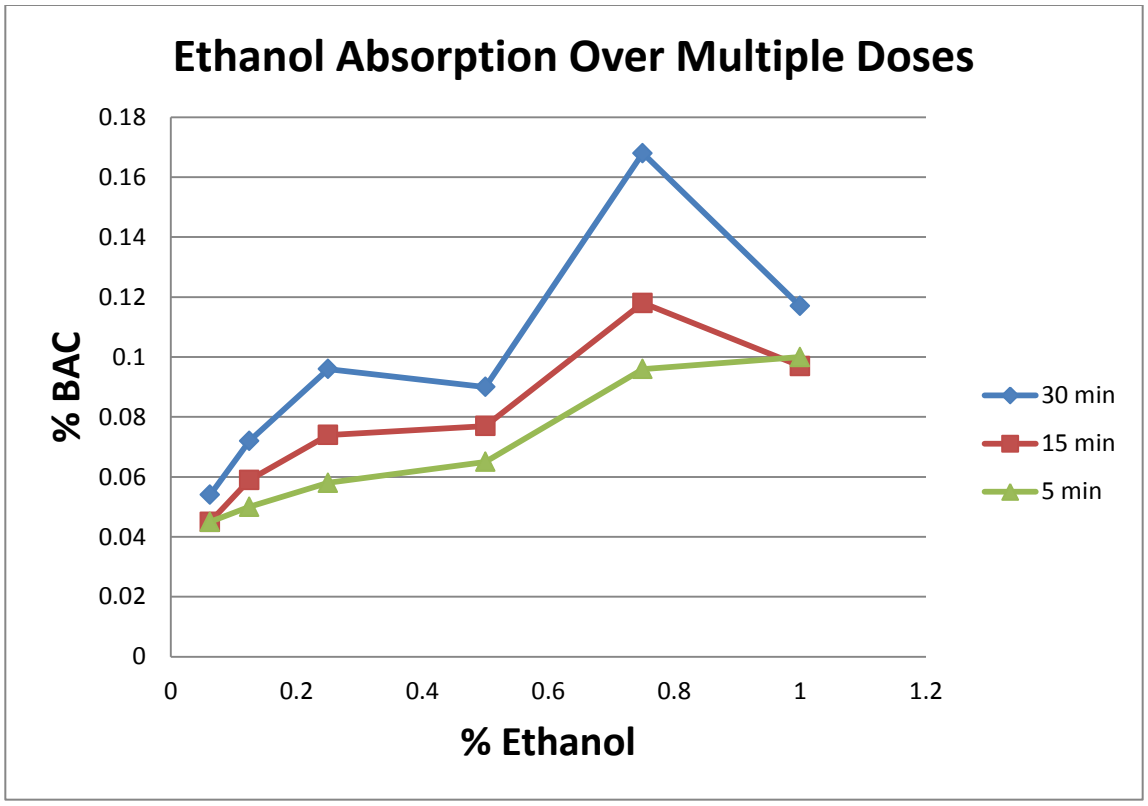

Figure 14. Ethanol absorption over time. Zebrafish are exposed to variable doses of ethanol (0.0625%, 0.125%, 0.25%, 0.5%, 0.75%, and 1.0%) for 5, 15, and 30 minute intervals.

When acute exposure is for five or 15 minutes, all doses (0.0625%, 0.125%, 0.25%, 0.5%, 0.75%, and 1.0%) followed a very similar pattern of absorption, with the smallest and highest doses for both time points nearly identical Figure 11. This stepwise pattern of alcohol uptake is expected and took place between the BAC levels of 0.04 and 0.1%. When acute exposure is for 30 minutes, doses 0.0625%, 0.125%, 0.25%, and 0.5% resulted in a similar absorption pattern compared to the shorter exposure times mentioned above, which resulted in BAC levels ranging from 0.05% and 0.09%. However, the larger doses (0.75%, and 1.0%) yielded a higher BAC level after 30 minutes of acute exposure. Specifically, after 30 minutes the 0.75% dose resulted in a BAC of 0.168% (the highest BAC of any dose or time point); this is in contrast to the 1.0% dose, which after 30

minutes resulted in a slightly smaller (but still large) BAC of 0.117%. The most interesting dose is the 0.75% alcohol and its conceivable effects on adult zebrafish behavior. It is possible that this drastic increase in BAC may alter a behavior that has not yet been assessed.

After demonstrating the zebrafish's ability to absorb ethanol, the researcher exposed subjects to various doses of ethanol on days nine and eleven of the trial phase of the novel associative learning task. Each subject received two of the four possible ethanol doses 0.0625%, 0.25%, 0.50%, and 0.75 of ethanol per volume during discrimination trials. Ethanol exposure took place on trial days nine and eleven with day ten remaining a normal trial to control for any possible detrimental effects of the ethanol exposure. A total of seventeen subjects are exposed to both 0.0625% and 0.50% ethanol by volume during trials day nine and eleven. Repeated measures ANOVA finds no significant behavioral effect from exposure to ethanol at the 0.0625% or 0.50% dose Figure 12. Acute ethanol exposure has no significant effect on trial performance nor does it affect baseline performance.

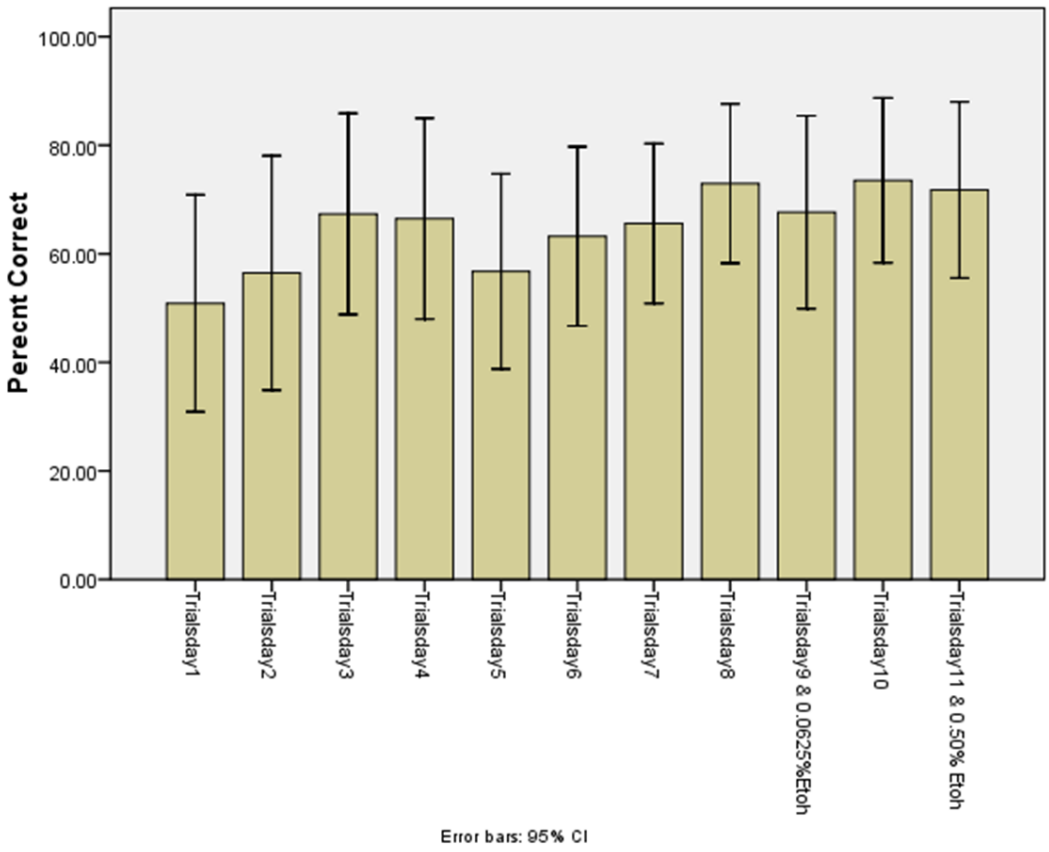

Figure 15. Acute ethanol exposure at the 0.0625% and 0.50% dose. A total of n=17 subjects are exposed to both 0.0625% and 0.50% ethanol by volume during trials day nine and eleven. Statistical analysis finds no significant behavioral effect from exposure to ethanol at the 0.0625% or 0.50% dose.

For the 0.25% and 0.75% ethanol doses, the experiment is run identical to the previous dose with the exception of sample size. Fifteen subjects are exposed to both 0.25% and 0.75% ethanol by volume. Repeated measures ANOVA finds no significant behavioral effect from exposure to ethanol at the 0.25% and 0.75% dose. Figure 13 as with the previous doses, there is no significant difference between trial performance prior neither to nor during ethanol exposure. Also, exposure to ethanol did not have an effect on trial performance post ethanol exposure.

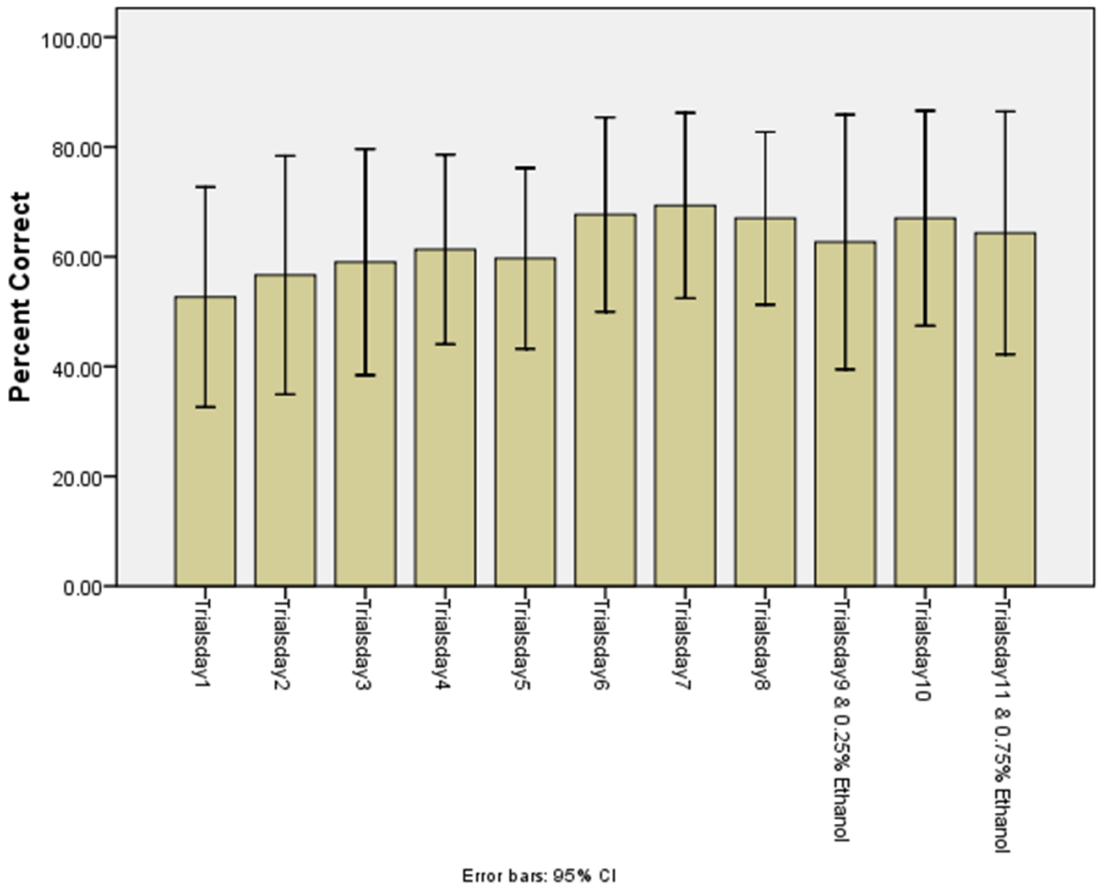

Figure 16. Acute ethanol exposure at the 0.25% and 0.75% dose. A total of n=15 subjects are exposed to both 0.25% and 0.75% ethanol by volume during trials day nine and eleven. Statistical analysis finds no significant behavioral effect from exposure to ethanol at the 0.25% and 0.75% dose.

Discussion

The novel associative learning task is developed in an effort to strengthen and further progress zebrafish as a behavioral model. The initial effort is to replicate the three choice appetite driven task as described by Bilotta et al. (2005). Though the Bilotta study is the framework for the current set of experiments, the original study is more a measure of visual acuity. The initial goal was to replicate the Bilotta study, yet were Bilotta stopped testing fish as soon as they reached criteria (80% correct) we wanted to test beyond the day of acquisition. However, after a concerted effort the researcher is unable

to replicate the task. The image below depicts the journey from attempting to replicate the three choice appetite discrimination task to the creation of the novel associative learning task.

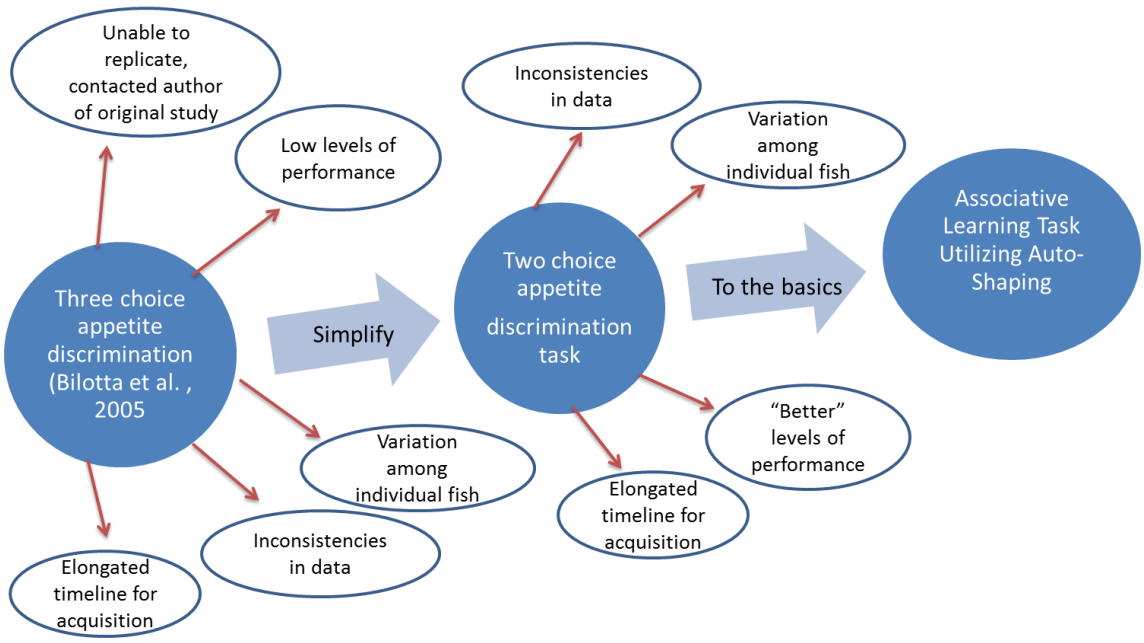

Figure 17. Origin of the task. A description of behavioral paradigms that influenced the development of the reported task.

During efforts to replicate the three choice appetite driven task of Bilotta et al. (2005), the researcher encountered several obstacles including: inability to replicate despite contacting the original author, inconsistencies in data, comparatively low levels of performance, and an elongated timeline for task acquisition. Where the Bilotta study saw performance at 80% correct in three to 23 days, efforts to replicate only reached 50% correct in approximately 26 days. Hoping to better understand the Bilotta study, the three choice task is simplified to a two choice task following the same protocol. Unfortunately, the experimenter observed similar difficulties with the two choice task. In light of the inability to replicate the Bilotta task, the researcher developed the novel associative

learning task using a parsimonious approach to understand the capacity for learning of individual zebrafish. The current task uses a similar apparatus to the three choice discrimination task, but improvements have been made to the apparatus which aid in removing any extraneous variables from the experiment. The gate was modified to fit within the partition to reduce water disruption, and is operated with a pulley system to prevent the presence of the experimenter over the testing apparatus.

One common factor both in the Bilotta and current study is the variation in learning between zebrafish. In the current study, variance in performance between subjects became a factor in assessing the capacity for learning in the zebrafish. Though performance variance appears to be a function of the capacity of individual zebrafish to acquire the conditioned response, several hypotheses are discussed to address variance. One hypothesis could be that the zebrafish were not properly habituated to the apparatus; yet, insufficient habituation would present as immobility or hyperactivity neither of which were observed in the study. The temperature and oxygen levels of the water could also be a source of variation in zebrafish behavior. Reduced oxygen levels would induce suspended animation, while a significant increase or decrease in water temperature can lead to a stress response or death (Malek, Sajadi, Abraham, Grundy, & Gerhard, 2004). To combat these confounds a heater and water circulator is placed in a rear compartment of the testing apparatus and the water is changed after each testing session. Though the argument could be made for multiple sources of variation in task performance, variance in performance may be primarily attributed to the variance in the zebrafish genome. The variance both between and within stains in the zebrafish genome is much greater than the variation observed in other vertebrate models (Guryev et al., 2006). The genetic variation

in zebrafish provides evidence for performance variance not being an adverse characteristic, but instead being a hallmark of the species as a behavioral model.

In an effort to assess the learning and acquisition capabilities, the researcher created a novel associative learning task. The task is based off of the Skinner box and operant conditioning. Through conditioning, subjects develop an association between a specific action and reward, which reinforces the conditioned operant response. The task was cultivated in an aim to produce a simplified and replicable visual association task that is valuable as a behavioral paradigm, and also has the capacity to be further developed.

The data from the novel associative learning task demonstrates both the ability of the subjects to learn the task as well as the ability of the task to be replicated. The task is run in four separate groups with two different researchers, yet there is no significant difference among trial performance of any group. One component that continued to come to light is variation among individuals. The researcher finds that during the trial phase, an individual zebrafish falls into one of three performance categories. The data for the majority of the subjects suggests that the conditioned response is created during the training phase. For these fish the trial phase acts merely to reinforce or strengthen the conditioned association. Zebrafish in this group at the onset of the trial phase present with a performance level above 60% with a large number of individuals in the group responding to the stimulus more than 80% of the time. The second smaller group includes subjects that perform at a lower level at the onset of the trial phase, but performance gradually improves over the eight days of trials. The data for these subjects represents what is typically considered as a learning curve. During the trial phase, the association

between stimulus and reward is not only strengthened, but also advanced in some cases. The third and smallest group consists of subjects that display low performance when compared to group one and failed to strengthen the association between stimulus and reward. The variation in the ability of individuals to make the association between stimulus and reward brings to light an aspect of using zebrafish in behavioral tasks which has previously not been characterized. Taking into account the lack of increased activity during the habituation and training phase, and the lack of increase performance during the trial phase, it is arguable that trial performance is dependent on the association between stimulus and reward which is conditioned during the training phase. The consistent level of trial performance after day two, as well as the lack of significant variation from one day to the next, provides evidence for the establishment of baseline performance. Regardless of the specific point at which the association takes place, the difference in performance levels provides evidence of a conditioned response. Zebrafish performance on the novel task is analogous to the conditioned response seen in the rodent and avian literature on similar tasks.

The zebrafish model is relatively new to behavioral research, and only recently have studies been performed with the goal of understanding capacity of zebrafish for use in behavioral paradigms. However, developmental and genetic studies have laid the ground work for pharmacological manipulation of homologous neurochemical and sensory systems between zebrafish and humans. The eye of the zebrafish is similar to humans, consisting of both cones and rods (Fadool & Dolwing, 2008). This similarity conceivably opens the door for comparisons between how zebrafish and humans perceive visual stimuli. One study demonstrates that the medial zone of the dorsal telencephic

region and the dorsal nucleus of the ventral telencephic area are involved in choice behaviors in the zebrafish (Lau et al., 2011). In zebrafish medial zone of the dorsal telencephic region and the dorsal nucleus of the ventral telencephic area are the anatomical homologs to the mammalian amygdala and striatum respectively (Lau et al., 2011). Several neurochemicals such as dopamine, serotonin, and acetylcholine can be found in both the rodent and zebrafish. However, zebrafish differ from rodents in that zebrafish cortisol, which is released during the stress response is measured using human salivary cortisol assays. Rodents on the other hand produce an analogous hormone.

The literature provides evidence for homology between zebrafish, rodents, and human, as well as the capability of using psychoactive compounds to elicit behavioral responses similar to what is observed in the rodent model. The effects of several pharmacological agents including monoamine oxidase inhibitors (MAOIs) and Lysergic acid diethylamide (LSD) on zebrafish are analogous to the behavioral effects seen in the rodent literature (Stewart et al., 2012). Zebrafish performance on the novel task is analogous to the conditioned response seen in the rodent, avian, and human literature on similar tasks. The simplistic design of the current task allows for the case to be made for analogy between the conditioned response of the zebrafish with similar conditioned responses in mammalian species.

Interestingly, acute ethanol exposure had no significant effect on performance across the four tested doses. Though the lack of effect does undermine the task as a useful behavioral screen, due to the relatively non-specific nature of ethanol, the researcher is optimistic that the task would be a useful screen when testing compounds with more specific neurochemical modulation. The data presented above provides compelling

evidence that within five minutes, which is the length of the habituation period before the onset of the discrimination trials, a significant amount of ethanol is being absorbed across all doses. Acute ethanol exposure for five minutes across doses (0.0625%, 0.125%, 0.25%, 0.5%, 0.75%, and 1.0%) results in corresponding blood alcohol concentrations of 0.045%, 0.05%, 0.058%, 0.065%, 0.096% and 0.1% respectively. Acute ethanol exposure for 30 minutes across doses (0.0625%, 0.125%, 0.25%, 0.5%, 0.75%, and 1.0%) results in corresponding blood alcohol concentrations of 0.054%, 0.072%, 0.096%, 0.09%, 0.168% and 0.117% respectively. With an average session lasting approximately 38 minutes, the data shows that within 30 minutes a significant concentration of ethanol is absorbed by the zebrafish.

The results of the ethanol treatment in the current study suggest that acute ethanol treatment is not sufficient to disrupt the conditioned response. The lack of effect provides evidences for: lack of disruption of sensory processing and visual cues, no obvious effect on locomotion, no appetite effect, and no disruption in learned association. While other zebrafish models have seen significant effects of acute ethanol exposure, all of these paradigms were novel tasks, which could explain the difference in observed ethanol effects (Echevarria et al., 2011). One could argue that the conditioned response is well learned and the non-specific effects of ethanol are not sufficient to disrupt the association. A future point of interest would be to expose zebrafish to ethanol during the habituation or training phases while the association between stimulus and reward is being made. The lack of effect with acute ethanol exposure does not assess the full capabilities of the task as a behavioral assay, and the researcher would expect to see a drug effect if a psychoactive compound with a more specific neurochemical modulation were tested.

The research presented in this manuscript along with the design of the apparatus allows researchers to take what has been learned from the current task and develop it into a more complex paradigm such as go/no-go.

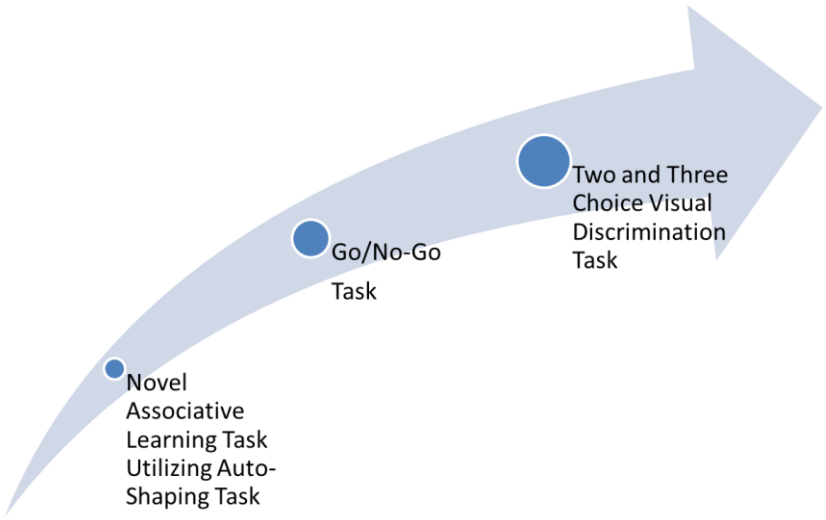

Figure 18. Possible progression of the task. The novel task being reported provides the opportunity for further development.

As further development of behavioral tasks for the zebrafish model is paramount for the establishment of the species as a behavioral paradigm, the chart above demonstrates the capacity of the current task for further expansion. Using what has been learned from the novel associative learning task, the most logical next step would be to adjust the protocol, which would effectively create a go/no-go task. Furthermore, other more ambitious paradigms are currently far from reach, and require additional knowledge of the behavioral characteristics of the species.

CHAPTER V

SUMMARY

The novel associative learning task was developed to access the species' capacity for learning. The study addresses three major goals. First, to establish a simple operant task based on a three choice discrimination test previously reported in the literature. The data presented above provide support for the parsimonious design of the current task as well as evidence for reliability of task performance based on the lack of variation between testing group. Second, a better understanding of zebrafish learning was gained with thorough analyses of all stages of testing: habituation, training, and discrimination trials. The significant performance increase in comparing day one of trials to days three, four, six, seven, and eight provides evidence for learning, and acquisition of the conditioned response. Third, to analyze any observed differences between individuals. Throughout the literature variability between subjects is sited as being a factor in zebrafish behavioral paradigms. In an aim to characterize these individual differences the current study analyzed subjects by grouping them according to performance levels. The data suggest that subjects with high levels of performance display both quicker rates of learning as well as more stable performance, while poor performers are not only slower learners but also present with more variable performance.

The research presented provides support for the novel association task, aids in gaining a better understanding of the learning processes, and identifies individual differences. The novel associative learning task differs from any present well established behavioral model and lends itself to future development. The task provides the zebrafish community with a high output behavioral task which is readily replicated and allows one

researcher to test between eight and ten fish over a period of four weeks with a total of sixteen days of actual testing. The sixteen day period consists of all three phases of testing: habitation, training, and discrimination trials. The future growth of behavioral research in zebrafish relies on the research community to develop new and more multifaceted behavioral paradigms. Behavioral models found in the rodent and avian literature can be used as a blue print to realize the full potential of the zebrafish species.

REFERENCES

Al-Imari, L., & Gerlai, R. (2008). Sight of conspecifics as reward in associative learning in zebrafish Danio rerio. *Behavioural brain research, 189*(1), 216-219.

Andrews, J.S., Jansen, J.H.M., Linders, S., Princen, A., & Broekkamp, C.L.E. (1995). Performance of four different rat strains in the autoshaping, two-object discrimination, and swim maze tests of learning and memory. *Physiology & behavior, 57*(4), 785-790.

Atnip, G. W. (1977). Stimulus-and response-reinforcer contingencies in autoshaping, operant, classical, and omission training procedures in rats. *Journal of the Experimental Analysis of Behavior, 28*(1), 59.

Bilotta, J., Pisner, M.L., Davis, E.C., & Haggbloom, S.J. (2005). Assessing appetitive choice discrimination learning in zebrafish. *Zebrafish, 2*, 259-268.

Burgess, H.A. & Granato, M. (2007) Modulation of locomotor activity in larval zebrafish during light adaptation. *Journal Experimental Biology, 210*, 2526-39.

Carvan, M. J. III, Loucks, E., Weberb, N.D., & Williams, F.E. (2004). Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis. *Neurotox Teratol, 2*6,757-768.

Colwill, R. M., Raymond, M. P., Ferreira, L., & Escudero, H. (2005). Visual discrimination learning in zebrafish (*Danio rerio*). *Behavioral Processes, 70*, 19-31.

Eagle, D.M., Bari, A., Robbins, T.W. (2008), The neuropsychopharmacology of action inhibition: cross-species translation of the stop-signal and go/no-go tasks. *Psychopharmacology (Berl),199*(3),439-56.

Echevarria, D.J., Hammack, C.M., Jouandot, D.J., & Toms, C.N. (2010). Does acute alcohol exposure modulate aggressive behaviors in the zerbrafish (Danio Rerio), or is the bark worse that the bite? *International Journal of Comparative Psychology, 23*, 62-69.

Echevarria, D. J., Jouandot, D. J., & Toms. C. N. (2011). Assessing attention in the zebrafish. Are we there yet? Progress in *Neuropsychopharmacology & Biological Psychiarty, 35*(6), 1416-1420.

Fadool, J. M., & Dowling, J. E. (2008). Zebrafish: a model system for the study of eye genetics. *Progress in retinal and eye research, 27*(1), 89-110.

Gerlai, R., Fernandes, Y., & Pereira, T. (2009). Zebrafish (Danio rerio) responds to the animated image of a predator: Towards the development of an automated aversive task. *Behavioural Brain Research, 201*, 318-324.

Guo, S. (2009). Using zebrafish to assess the impact of drugs on neural development and function. *Expert Opinion in Drug Discovery, 4*(7), 715-726.

Guryev, V., Koudijs, M. J., Berezikov, E., Johnson, S. L., Plasterk, R. H., van Eeden, F. J., & Cuppen, E. (2006). Genetic variation in the zebrafish. *Genome research, 16*(4), 491-497.

Johnson, A., & Crowe, D.A. (2008). Revisiting Tolman, his therories and cognitive maps. *Cognitive Critique, 1*, 43-72.

Kabashi, E., Brustein, E., Champagne, N., & Drapeau, P. (2011). Zebrafish models for the functional genomics of neurogenic disorders. *Biochimica et Biophysica, 1812*, 335-345.

Kimmel, C.B., Ballard W.W., Kimmel S.R., Wullmann B., & Schilling T.F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics, 203*, 253-310.

Lau, B., Bretaud, S., Huang, Y., Lin, E., & Guo, S. (2006). Dissociation of food and opiate preference by a genetic mutation in zebrafish. *Genes, Brain and Behavior, 5*, 497-505.

Lau, B. Y., Mathur, P., Gould, G. G., & Guo, S. (2011). Identification of a brain center whose activity discriminates a choice behavior in zebrafish. *Proceedings of the National Academy of Sciences*, *108*(6), 2581-2586.

Levin, E.D. (2011). Zebrafish assessment of cognitive improvement and anxiolysis: filling the gap between in vitro and rodent models for drug development. *Reviews in the Neurosciences, 22*, 75-84.

Levin, E. D., Chrysanthis, E., Yacisin, K., & Linney, E. (2003). Chlorpyrifos exposure of developing zebrafish: effects on survival and long-term effects on response latency and spatial discrimination. *Neurotoxicology and Teratology, 25*, 51-57.

Malek, R. L., Sajadi, H., Abraham, J., Grundy, M. A., & Gerhard, G. S. (2004). The effects of temperature reduction on gene expression and oxidative stress in skeletal muscle from adult zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *138*(3), 363-373.

Morse, W. H., & Skinner, B. F. (1957). A second type of superstition in the pigeon. *American Journal of Psychology, 70*, 308-311.

Peterson, G.B. (2004). A day of great illumination: B. F. Skinner's discovery of shaping. *Journal of the Experimental Analysis of Behavior, 82*, 317-328.

Rico, E. P., Rosemberg, D. B., Seibt, K. J., Capiotti, K. M., Da Silva, R. S. & Bonan, C. D. (2011). Zebrafish neurotransmitter systems as potential pharmacological and toxicological targets. *Neurotoxicology, 33*, 608-617.

Rink, E., & Guo, S. (2004). The too few mutant selectively affects subgroups 1 of monoaminergic neurons in the zebrafish forebrain. *Neuroscience, 127*, 147-154.

Rinkwitz, S., Mourrain, P., & Becker, T. S. (2011). Zebrafish: an integrative system for neurogenomics and neurosciences. *Progress in Neurobiology, 93*, 231-243.

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., ... & Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural brain research, 205*(1), 38-44.

Saverino, C., & Gerlai, R. (2008). The social zebrafish: Behavioral responses to conspecific, heterospecific, and computer animated fish. *Behavioural BrainResearch, 191*(1), 77-78.

Siegel, G.J., Agranoff, B.W., & Albers, R.W. (1999). MPTP-Induced Parkinsonian Syndrome. *Basic Neurochemistry: Molecular, Cellular and Medical Aspects*. 6th edition. http://www.ncbi.nlm.nih.gov/books/NBK27974/

Sison, M., & Gerlai, R. (2010). Associative learning in zebrafish (Danio rerio) in the plus maze. *Behavioral Brain Research, 207*, 99-104.

Skinner, B.F. (1948). Superstition in the Pigeon. *Journal of Experimental Psychology*, *38*, 168-172.

Skinner, B. F. (1963). Operant behavior. *American Psychologist, 18*, 503-515.

Skinner, B. F., Ferster, C. B., & Ferster, C. B. (1997). *Schedules of reinforcement*. Acton, MA: Copley Publishing Group.

Spence, R., Gerlach G., Lawrence C., & Smith C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biological Reviews, 83*, 13–34.

Staddon, J. E. R., & Cerutti, D. T. (2003). Operant conditioning. *Annual review of psychology*, *54*, 115.

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., & Kalueff, A. V. (2012). Modeling anxiety using adult zebrafish: A conceptual review. *Neuropharmacology*, *62*(1), 135-143.

Tolman, E.C. (1948). Cognitive maps in rats and men. *Psychology Review, 55*, 189–208.

White, R.M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., Bourque, C., & Zon, L.I. (2008). Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. *Cell Stem Cell*, *2*(2), 183-189.